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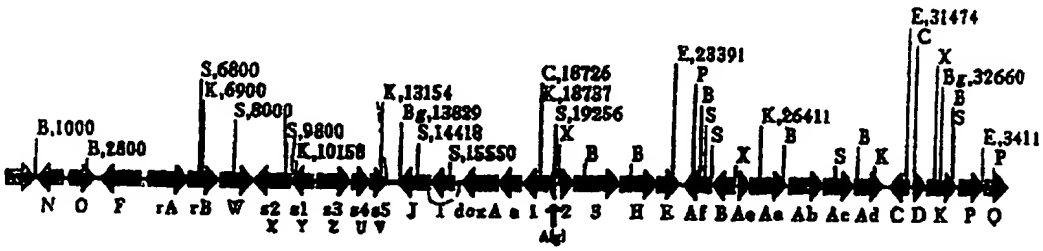

(57) Abstract

The present invention provides novel methods for producing doxorubicin using daunomycin as a substrate. One method employs a genetically engineered host microorganism which is transformed with a vector, preferably a plasmid, which contains the *doxA* gene. Preferably, the *doxA* gene, also referred to herein as "*doxA*", is cloned into a plasmid which is then introduced into the host microorganism, preferably a bacterial host, more preferably *Streptomyces*, to provide a transformed host microorganism. The *doxA* gene, when present on a plasmid, confers on the transformed host the ability to convert daunomycin and 13-dihydrodaunomycin, to doxorubicin. The *doxA* gene encodes a P450-like enzyme which catalyzes the hydroxylation of daunomycin and 13-dihydrodaunomycin at C-14 to form doxorubicin; such enzyme is designated "daunomycin C-14 hydroxylase". Thus, the expression of *doxA* in the transformed host using a plasmid which contains *doxA* enables the transformed host to convert daun mycin to doxorubicin. The doxorubicin is then extracted from host microorganism cultures.

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METHODS OF PRODUCING DOXORUBICIN

Background of the Invention

Daunomycin and doxorubicin are clinically important chemotherapeutic agents. Daunomycin is used primarily to treat adult myelogenous leukemia. Doxorubicin is widely used to treat a variety of neoplasias, making it the more valuable of the two anticancer drugs. The world wide market for doxorubicin is estimated to exceed $156 million. As of 1984, the wholesale price for doxorubicin was estimated to be $1,370,000 per kilogram.

While daunomycin is synthesized by several species of *Streptomyces*, doxorubicin is biologically synthesized by only one strain, a mutant strain of *Streptomyces peucetius*, called *S. peucetius subsp. caesius* which is available from the American Type Culture Collection under Accession number 27952.

The alternative *in vitro* laboratory synthesis of doxorubicin is difficult. The *in vitro* synthesis of doxorubicin is a process involving multiple steps and resulting in a poor yield, with a lack of stereospecificity in several of the synthetic steps, producing forms which are difficult to separate.

Chemical synthetic procedures are known for converting daunomycin to doxorubicin; however they require the use of halogens in the synthetic process.

It would be desirable to have an efficient, cost-effective method for producing doxorubicin that does not require the use of halogens in the synthetic process.

Summary of the Invention

The present invention provides novel methods for producing doxorubicin using daunomycin as a substrate. One method employs a genetically engineered host microorganism which is transformed with a vector, preferably a plasmid, which contains the *doxA* gene. Preferably, the *doxA* gene, also referred to herein as "*doxA*", is cloned into a plasmid which is then introduced into the host microorganism, preferably a bacterial host, more preferably *Streptomyces*, to provide a transformed host microorganism. The *doxA* gene, when present on a plasmid, confers on the transformed host the ability to convert daunomycin and 13-dihydrodaunomycin, to doxorubicin. The *doxA* gene encodes a cytochrome P450-type enzyme

which catalyzes the hydroxylation of daunomycin and 13-dihydrodaunomycin at C-14 to form doxorubicin; such enzyme is designated "daunomycin C-14 hydroxylase". Thus, the expression of *doxA* in the transformed host using a plasmid which contains *doxA* enables the transformed host to convert daunomycin to doxorubicin. The doxorubicin is then extracted from host microorganism cultures.

Another method for producing doxorubicin involves incubating the daunomycin C-14 hydroxylase with daunomycin, then extracting the doxorubicin from the solution.

Another method involves adding daunomycin to cultures of *Streptomyces* sp. strain C5 and extracting doxorubicin from the culture fluid and the host cells.

The invention also relates to daunomycin C-14 hydroxylase, novel plasmids, novel polylinkers and novel transformed host microorganisms employed in such method for producing doxorubicin. The invention also relates to methods for producing anthracyclines, such as 13-deoxycarminomycin and 13-deoxydaunomycin, 13-dihydrocarminomycin and 13-dihydrodaunomycin, carminomycin and daunomycin.

Brief Description of Figures

Figure 1 is a restriction map of the *Streptomyces* sp. strain C5 daunomycin biosynthesis gene cluster which shows the position of *doxA* within the cluster. Abbreviations for restriction endonuclease sites are as follows: "B" represents *BamHI*; "Bg" represents *BglII*; "C" represents *ClaI*; "E" represents *EcoRI*; "K" represents *KpnI*; "P" represents *PstI*; "S" represents *SstI*; and "X" represents *XhoI*;

Figure 2 is a detailed restriction map of part of the daunomycin biosynthesis gene cluster from *Streptomyces* sp. strain C5. Abbreviations for restriction endonuclease sites are as follows: "B" represents *BamHI*; "Bg" represents *BglII*; "E" represents *EcoRI*; "K" represents *KpnI*; "P" represents *PstI*; "S" represents *SstI*; "Sp" represents *SphI*;

Figure 3 is a nucleotide sequence of the 3196 base pair *KpnI-SstI* DNA fragment from *Streptomyces* sp. strain C5 containing the *doxA* gene. The deduced amino acid sequence of the daunomycin C-14 hydroxylase is given below the nucleotide sequence. Potential ribosome binding sites, designated "rbs" are identified, as are significant restriction endonuclease sites. The sequences and deduced products of the 3' end of *orf1*, all of *orfA*, and the 5' end of *dauI* are also shown;

Figure 4 shows the plasmid maps of plasmid pANT849 and the plasmids pANT42 and pANT842 which were used to construct pANT849;

Figure 5 shows the plasmid maps of pANT195 and plasmids pANT849, pANT186, pANT185, pANT235 and pUC19 all of which were used to construct pANT195;

Figure 6 shows the sequence of *snpR*, *doxA*, and the intervening sequences within plasmid pANT195;

Figure 7 shows plasmid maps of pANT192 and pANT193;

Figure 8 shows plasmid maps of pANT194 and pANT196;

Figure 9 shows the N-terminal, modified region of the *doxA* fusion protein;

Figure 10 shows plasmid maps of pANT198 and pANT199; and

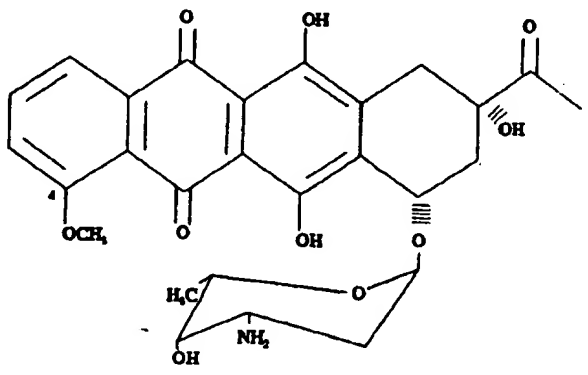
Figure 11 shows sequence of the *doxA* gene and upstream *melC1* promoter regeion in pANT196; and

Figure 12 shows a plasmid map of pANT144.

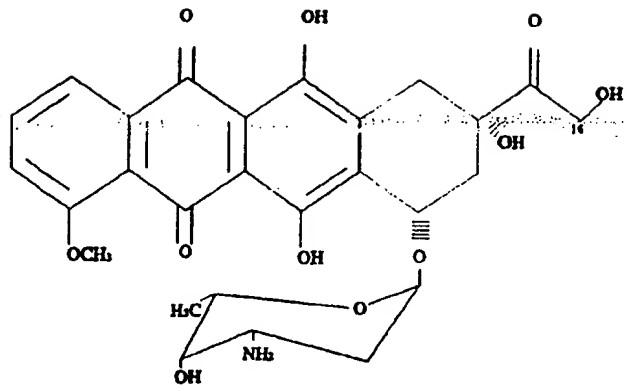
Detailed Description of Invention

A novel method for producing doxorubicin from daunomycin has been developed which employs genetically engineered host microorganisms that contain and express the gene *doxA*. Preferably, *doxA* is cloned into a plasmid which is then inserted into a host microorganism, preferably a bacterial host, more preferably *Streptomyces*, most preferably *Streptomyces lividans* to provide a transformed host. The *doxA* gene encodes daunomycin C-14 hydroxylase which catalyzes the hydroxylation of daunomycin and 13-dihydrodaunomycin at C-14 to form doxorubicin. Thus, expression of *doxA* in the transformed host using a plasmid which contains the *doxA* gene enables the transformed host to convert daunomycin to doxorubicin.

Daunomycin is also known as daunorubicin; doxorubicin is also known as 14-hydroxydaunomycin and adriamycin. The structure of daunomycin is shown below:



The structure of doxorubicin is shown below:



Cloning and Analysis of the *DoxA* Gene

Streptomyces sp. strain C5 synthesizes several compounds in fermentations, that is such compounds are produced from common metabolic intermediates and without the addition of precursor anthracycline molecules to the culture media. *Streptomyces* sp. strain C5 produces the following anthracyclines: ϵ-rhodomycinone; daunomycin; 13-dihydrodaunomycin; baumycin A1; and baumycin A2. Nevertheless, a gene, the *doxA* gene, was discovered in the genome of *Streptomyces* sp. strain C5 which, when expressed, converts daunomycin, particularly exogenous daunomycin, to doxorubicin. Preferably, the conversion of daunomycin to doxorubicin is accomplished by cloning the *doxA* gene along with a promoter into a plasmid which is then introduced into a host microorganism.

It has also been discovered that *Streptomyces* sp. strain C5 can convert small amounts, less than 10%, daunomycin to doxorubicin in the absence of plasmid containing the *doxA* gene.

Preferably, the *doxA* gene is cloned from *Streptomyces*, preferably *Streptomyces* sp. strain C5. Alternatively, the *doxA* gene is synthesized using conventional oligonucleotide synthesis techniques and equipment.

The *doxA* gene is located in the daunomycin biosynthesis gene cluster between the daunomycin polyketide biosynthesis genes and *dauI*, a putative transcriptional activator as shown in Figure 1. The location of *doxA* within the *Streptomyces* sp. strain C5 daunomycin biosynthesis gene cluster is shown in Figure 2.

The approximately 8 kbp region between *dauI*, a gene encoding an activator regulatory protein for daunomycin biosynthesis, and the daunomycin polyketide synthase biosynthesis genes was sequenced in its entirety.

Plasmids containing inserts to be sequenced were isolated from recombinant *E. coli* JM83, available from Dr. Mary Berlyn, *E. coli* Genetic Stock Center, Yale University, P.O. Box 6666, New Haven, Ct 06511-7444 by the methods disclosed in Carter, M.J., and I.D. Milton, (1993), "An Inexpensive and Simple Method for DNA Purification on Silica Particles," *Nucleic Acids Res.* Volume 21, p. 1044. The *doxA* DNA was sequenced in both directions, that is, both strands were sequenced using Sequenase enzyme, Version 2.0 from the United States Biochemical Corp., Cleveland, Ohio, according to the manufacturer's instructions, and as described in Ye, et. al., 1994, "Isolation and Sequence Analysis of Polyketide Synthase Genes from the Daunomycin-producing *Streptomyces* sp. strain C5" *J. Bacteriol.* 176:6270-6280. Doubled-stranded DNA templates were employed. The terminated chains were labeled with 3000 Ci/mmol (α-^{32}P)dCTP from Dupont-New England Nuclear, Boston, MA. The terminated labeled chains were separated on a 6% weight-to-volume polyacrylamide gel containing 10% (volume-to-volume) formamide and visualized by autoradiography. Sequencing reactions were carried out using 7-deaza-dGTP nucleotide mixes to reduce compressions. Forward (-40) and reverse universal pUC/m13 17-mer oligonucleotide primers from U.S. Biochemical Corp. were used to obtain the initial sequences in the inserts. Specific primers, 15-mer oligonucleotides, were generated based on sequencing results for extension of the sequences within the inserts.

DNA sequence data were analyzed using Clone Manager from Stateline, PA, and the Sequence Analysis Software Package of the Genetics Computer Group from Madison, WI.

The nucleotide sequence between *dauI* and the ketoreductase just downstream of *dauA-orfG* is shown in Figure 3. Two complete open

reading frames, *orfA* and *doxA*, were found within this sequence; *OrfA* encodes a protein of M_r 28,808, and 275 amino acid residues, and *doxA* encodes a protein of M_r 46,096 and 422 amino acid residues.

Plasmids

The *doxA* gene is inserted into a vector, preferably a plasmid. Optionally, the plasmid contains genes from the daunomycin synthesis cluster in addition to *doxA*. However, preferred plasmids lack *dauA*(g) and more preferred plasmids lack *dauA*(g), *orf1* and *orfA*.

The preferred plasmids contain not only the translated portion of *doxA* but a promoter. Suitable promoters include, *Streptomyces* promoters for example, *melC1*-P, *ermE*-P, wild type, and *snpA*-P. The *snpA*-P promoter is the most preferred. Preferably, the promoter is a protein activated promoter, and most preferably, an SnpR-activated promoter. Less preferred plasmids, such as pANT196, contain a *melC1* promoter from pIJ702 for expression of *doxA*. Also less preferred are plasmids which lack a known promoter, such as pANT194.

The most preferred plasmid which contains *doxA* is designated "pANT195" which is shown in Figure 5. Host microorganisms, when transformed with plasmid pANT195, convert 100% daunomycin to doxorubicin. Other plasmids which contain *doxA* are suitable, including, for example pANT192, pANT193, pANT194 and pANT196. Host microorganisms, when transformed with plasmid pANT192, typically convert about 25% daunomycin to doxorubicin at a concentration of 2 μg/ml. Host microorganisms when transformed with pANT193 convert about 80% daunomycin to doxorubicin and about 20% daunomycin to 13-dihydrodaunomycin, at a daunomycin concentration of 2 μg/ml.

Construction of the Plasmids

Digestion of and ligation of DNA was performed using conventional techniques described by Maniatis et al. (1982) in "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory, N.Y.

Construction of pANT195

Plasmid pANT195, shown in Figure 5, has about 7.04 kbp of DNA. Plasmid pANT195 was constructed by inserting the 1.72 kbp *SphI-SacI* fragment insert containing intact *doxA* from plasmid pANT186 into pANT849.

First, plasmid pANT186 was constructed by constructing pANT235. Plasmid pANT235 is described in Ye et. al. 1994 "Isolation and Sequence Analysis of Polyketide Synthase Genes from the Daunomycin-Producing *Streptomyces* sp. Strain C5" *J. Bacteriol.* Vol. 176, pp. 6270-6280. Plasmid pANT235 is a 9.2 kbp plasmid which contains a 6.48 kbp *BamHI-BglII* DNA fragment from the *Streptomyces* sp. strain C5 daunomycin biosynthesis gene cluster. The *doxA* gene lies within the insert of pANT235 which is derived from the daunomycin biosynthesis gene cluster.

The *BamHI-BglII* DNA fragment had been cloned into the *BamHI* site of pUC19 to generate pANT235. Plasmid pUC19 is available from Gibco BRL, Gaithersburg MD.

Next, pANT235 was digested with *SalI* and *SstI* and the digestion products were purified on an agarose gel. The 1.67 kbp *SalI-SstI* fragment containing the 3' end of the *doxA* gene and the 5' end of *dauI* was extracted from the agarose gel and ligated into pUC19 with T4 DNA ligase, from Gibco BRL, Gaithersburg MD, to generate pANT185, as shown in Figure 5.

Next, pANT235 was used as the template for the polymerase chain reaction amplification of the 5'-end of the *doxA* gene containing an upstream ribosome binding site and *SphI* restriction site for the 5' end and *BspEI* restriction site for the 3' end.

The forward primer used in the polymerase chain reaction amplification of the *doxA* gene had the following nucleotide sequence:
5'-GACATGCATGCGGAGGGGTGCCTC-3' SEQ.ID 1
The forward primer which is used for the 5'-end, contains an *SphI* site with five extra nucleotides on the end and the extra ribosome binding site "GGAGG". The reverse primer had the following nucleotide sequence:

5'-GACGCAGCTCCGGAACGGGG-3' SEQ.ID 2

The reverse primer which is used for the 3'-end, has a *BspEI* site plus eight extra nucleotides.

The polymerase chain reaction amplification was carried out for 25 cycles using Deep Vent Polymerase from New England Biolabs, Beverly, MA. The solution for PCR included: 2.0 µl dimethylsulfoxide; 14.5 µl double distilled water; 1.25mM dNTPs, 16.0 µl of a total stock containing dATP, dCTP, dTTP, dGTP; 5.0 µl 10X Deep Vent Buffer, from New England Biolabs; 5 µl forward primer; 5 µl reverse primer; 0.5 µl Deep Vent polymerase; 2.0 µl DNA template, 14.5 µl distilled water, and boiled for 10 minutes. PCR was carried out by incubating the reaction mixture at 94°C in the absence of Deep Vent Polymerase for 5 minutes, following by 25 cycles of the following regimen: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds. After 25 cycles were completed, the mixture was incubated at 72°C for 7 minutes and then held at 4°C until further use.

The products of PCR were separated on a 0.8% agarose gel, and a 298 base pair DNA fragment was eluted from the gel. The 298 base pair DNA fragment was then digested with *SphI* and *BspEI* to generate a 285 base pair fragment with "sticky" ends. pANT185 was digested with *SphI* and *BspEI* and the 285 base pair fragment ligated into pANT185 to generate pANT186 which was introduced into *dam/dcm*-minus *E. coli* strain ET12567. MacNeil, et. al. (1992) "Analysis of *Streptomyces avermitilis* Genes Required for Avermectin Biosynthesis Utilizing a

Novel Integration Vector", *Gene*, vol. 111, pages 61-68. Plasmid pANT186 contains the complete *doxA* gene, upstream of which lay the newly constructed ribosome binding site having the nucleotide sequence GGAGG. The nucleotide sequence of the PCR-generated 5' end of the gene was confirmed by dideoxy sequencing.

Plasmid pANT849, shown in Figure 4, was constructed by first constructing pANT842. Plasmid pANT42, described in Lampel, et. al., 1992 "Cloning and Sequencing of a Gene Encoding a Novel Extracellular Neutral Proteinase from *Streptomyces* sp. Strain C5 and Expression of the Gene in *Streptomyces lividans* 1326" J. Bacteriology 174:2797-2808, was digested with *Kpn*I and religated, removing a 1.95 kbp *Kpn*I fragment to yield pANT842.

A novel polylinker sequence, having 48 nucleotides, was constructed according to conventional techniques using synthesized DNA oligonucleotides by Integrated DNA Technologies, Inc., Coralville, Iowa. The polylinker sequence has the following nucleotide sequence:

*Sph*I *Bgl*II *Sac*I *Dra*I *Hpa*I

GCATGCGAATTCAGATCTAGAGCTCAAGCTTTAAACTAGTTAACGCGT SEQ.ID 3

*Eco*RI *Xba*I *Hin*dIII *Spe*I *Mlu*I

Plasmid pANT842 was digested with *Sph*I-*Mlu*I to remove a 1.42 kbp *Sph*I-*Mlu*I fragment. The polylinker sequence was ligated into *Sph*I-*Mlu*I-digested pANT842 to provide plasmid pANT849. Plasmid pANT849, shown in Figure 4, has 5.34 kbp of DNA and lacks the *snpA* gene and most of *melC2*. Plasmid pANT849 does have the SnpR-activated *snpA*-promoter, which is located immediately upstream of the polylinker sequence as shown in Figure 4. pANT849 is a high copy number plasmid and contains the thiostrepton resistance gene as the selectable marker.

Next, to construct pANT195, a clone of pANT186 which contains the modified *doxA* gene, was digested with *Sph*I and *Sst*I, and pANT849 was digested with *Sph*I and *Sst*I. The fragment from pANT186 containing the *doxA* gene was ligated into the polylinker sequence of pANT849 to make pANT195 as shown in Figure 5.

The sequence of the region of pANT195 containing the *snpR* activator gene, the SnpR-activated *snpA* promoter, and the 5'-end-modified *doxA* gene is shown in Figure 6, and Seq. Id. No. 6.

Construction of pANT192

Plasmid pANT192 shown in Figure 7 is an 11.84 kbp plasmid which contains DNA encoding the acyl carrier protein and its putative promoter, a ketoreductase (*orf1*), *orf2*, a partial *orf3*, *orfA*, *doxA*, *dauI*, and most of *dauJ*. Plasmid pANT192 was constructed by removing the 6.52 kbp *Hin*dIII-*Eco*RI fragment from pANT235 which includes the entire *Bgl*II-*Bam*HI fragment, by digesting pANT235 with *Hin*dIII and *Eco*RI. Next pANT849 was digested with *Hin*dIII and *Eco*RI and the 6.52 kbp *Hin*dIII-*Eco*RI fragment from pANT235 was ligated into pANT849.

Construction of pANT193

Plasmid pANT193, shown in Figure 7, has 10.28 kbp of DNA and contains part of *orf1*, all of *orfA*, and *doxA* driven by the *snpR*-activated *snpA*-promoter. Plasmid pANT193 was constructed by digesting plasmid pANT235 with *Kpn*I, and the 1582 base pair *Kpn*I fragment removed. The plasmid was re-ligated to itself to form pANT235-k. Plasmid pANT235-k was digested with *Eco*RI and *Hin*dIII to remove the 4.95 kbp *Eco*RI-*Hin*dIII fragment. pANT849 was digested with *Eco*RI and *Hin*dIII and the 4.95 kbp *Eco*RI-*Hin*dIII fragment from pANT235-k was ligated into the digested pANT849.

Construction of pANT194

Plasmid pANT194, shown in Figure 8, has 8.97 kbp of DNA and contains the part of *orf1*, all of *orfA*, *dauI*, *dauJ* and *doxA* but lacks any known promoter to drive the expression of *doxA*. Plasmid pANT194 was constructed by digesting pANT192 with *Kpn*I to remove a 2.87 kbp *Kpn*I fragment and then religating the plasmid to itself.

Construction of pANT196

Plasmid pANT196, shown in Figure 8, has 7398 bp of DNA and possesses a promoter *melC1* which drives the expression of the *doxA* gene. pANT186 was digested with *Sph*I and *Sst*I and a 1712 nucleotide *Sph*I-*Sst*I fragment from pANT186 containing *doxA* was isolated and ligated into *Sph*I-*Sst*I digested pIJ702. Plasmid pIJ702 is a 5.686 kbp plasmid which is described in Katz, E., et. al. (1983) "Cloning and Expression of the Tyrosinase Gene from *Streptomyces antibioticus* in *Streptomyces lividans*" J. Gen. Microbiol. volume 129, pages 2703-2714.

Construction of pANT198

Plasmid pANT186 was digested with *Sph*I and then incubated with T4 DNA polymerase from Gibco BRL, according to the manufacturer's instructions to yield a blunt end. Plasmid pZero from Invitrogen, San Diego, Ca., was digested with *Eco*RI and then filled in 5' to 3' using Klenow fragment of DNA polymerase according to the manufacturer's instructions to provide a blunt end. Both fragments were purified according to the methods described in Carter, M.J. and I.D. Milton (1993), Nucleic Acids Res. volume 21, pages 1044. The fragments were precipitated in ethanol for one hour at -70°C and then digested with *Sst*I overnight. The plasmid and insert, each of which contains a single blunt end and an *Sst*I end, were purified from an agarose gel and then ligated overnight with T4 DNA ligase at room temperature, to provide pANT198.

Construction of pANT199

Plasmid pANT198 was digested with *Eco*RI-*Hin*dIII, the *Eco*RI-*Hin*dIII fragment removed and ligated into pTrcHisC from Invitrogen to construct pANT199. In pANT199, the *doxA* gene is translationally fused

with a leader sequence encoding six histidine residues, shown in SEQ. ID. NO. 7 so that the fusion protein can be affinity purified on a nickel-agarose gel.

pANT849

Plasmid pANT849 in addition to being useful to construct pANT195 is also useful expression vector for other genes. To construct other such plasmids pANT849 is digested with at least one restriction endonuclease corresponding to the restriction sites in the polylinker, such as, for example, *Sph*I, *Bgl*II, *Sac*I, *Dra*I, *Hpa*I, *Eco*RI, *Xba*I, *Hind*III, *Spe*I, or *Mlu*I. The desired gene sequence to be inserted into the plasmid is provided with sticky ends corresponding to the sticky ends of the cut pANT849. The desired gene is then ligated into the plasmid to provide a new plasmid derived from pANT849.

Host Microorganisms

Suitable host microorganisms for the *doxA* plasmid possess electron donating, cytochrome P450 accessory proteins; suitable accessory proteins include for example, NADPH:ferredoxin oxidoreductase and ferredoxin. The preferred host microorganisms are bacteria, more preferably *E. coli or Streptomyces* spp., most preferably *Streptomyces lividans* TK24 and *Streptomyces coelicolor* CH999. *Streptomyces coelicolor* CH999 is a mutant of *Streptomyces coelicolor* A3(2). *Streptomyces lividans* TK4 is available from Professor David A. Hopwood, Head, Department of Genetics, John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, United Kingdom. *Streptomyces coelicolor* CH999 is available from C. Khosla, Stanford University, Palo Alto, California and Professor David A. Hopwood. *S. peucetius* is available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, USA under the accession number 29050. *Streptomyces* sp. strain C5 was obtained from the Frederick Cancer Research Center, Frederick MD. *Streptomyces* sp. strain C5 is also available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, USA under the Accession number ATCC 49111.

Transformation of Host Microorganism

The plasmids are introduced into the host microorganism using conventional techniques. For example, *Streptomyces* spp. are transformed using electroporation as described in Pigac and Schrempf (1995) "A Simple and Rapid Method of Transformation of *Streptomyces rimosus* R6 and Other Streptomycetes by Electroporation", Appl. Environ. Microbiol. vol. 61, pages 352-356, or by protoplast transformation. *Streptomyces* are preferably transformed using protoplast transformation as described in Hopwood, et. al. (1985), "Genetic Manipulation of *Streptomyces*: A Laboratory Manual", The John Innes Foundation, Norwich, UK. *E. coli* strains are transformed using conventional transformation procedures as described in Maniatis, et al. (1982)

"Molecular Cloning: A Laboratory Manual" Cold Spring Harbor Laboratory, N.Y.

Plasmid pANT195 was introduced into *Streptomyces lividans* TK24 by protoplast transformation according to the procedures described in Hopwood et. al (1985), "Genetic Manipulation of *Streptomyces*: A Laboratory Manual", The John Innes Foundation, Norwich, UK. 500 µl of *Streptomyces lividans* TK24 protoplasts, were transformed with 10 µl of plasmid DNA, about 0.5 µg total, in 500 µl of T buffer for two minutes. The reaction was stopped with 500 µl of P buffer and the protoplasts were pelleted twice in a microcentrifuge for 7 seconds each spin. The pellets were then resuspended in 100 µl of P buffer and plated onto R2YE medium using a soft R2YE agar overlay with 50 µg/ml of thiostrepton added 24 hours later. The transformed microorganisms were tested for their ability to carry out daunomycin C-14 oxidation.

S. peucetius ATCC 29050, *Streptomyces coelicolor* CH999 and *Streptomyces* sp. strain C5 were transformed with plasmids pANT195 and pANT849 by protoplast transformation.

Daunomycin C-14 Hydroxylase

The daunomycin C-14 hydroxylase encoded by *doxA* is a cytochrome P450-type enzyme having a deduced *Mr* of 46,096. Daunomycin C-14 hydroxylase is a monooxygenase which inserts a single oxygen at carbon 14 on daunomycin. The daunomycin C-14 hydroxylase also appears to catalyze the two step oxidation at C-13 from methylene to hydroxyl to a keto functional group. Daunomycin C-14 hydroxylase also oxidizes 13-dihydrocarminomycin to carminomycin and 13-dihydrodaunomycin to doxorubicin.

The deduced amino acid sequence of daunomycin C-14 hydroxylase which is encoded by *doxA* of strain C5 is shown in Figure 3 and SEQ. ID. No. 5.

Preparation of Daunomycin C-14 Hydroxylase

Example A

The daunomycin C-14 hydroxylase was isolated and partially purified and subjected to spectrophotometric analysis. First, *S. lividans* TK24 strains containing plasmid pANT195 were grown in YEME medium containing 10 µg/ml thiostrepton for 48 hours at 30°C, harvested and washed by centrifugation and then broken in 100 mM, pH 7.5 sodium phosphate buffer using a French pressure cell at 15,000 lb/in^2. The cell debris and unbroken mycelia were pelleted by centrifugation at 10,000 × *g* for 30 minutes at 4°C, after which the supernatant was analyzed by visible spectrometry. The cytochromes within the supernatant derived from the cultures were reduced by a few grains of sodium dithionite. The supernatant samples were bubbled with carbon monoxide for 1 minute prior to analysis. Spectra were obtained using

a Beckman DU-64 single beam spectrophotometer and reduced-plus-CO minus reduced difference spectra were obtained by electronic subtraction.

Reduced-plus-CO minus reduced difference spectra of samples derived from cultures of *S. lividans* TK24 containing plasmid pANT195 revealed a peak at 450 nm, characteristic of cytochrome P450 enzymes. Such peak was not observed in samples derived from the control culture, which lack the *doxA* gene.

Proteins derived from both *S. lividans* TK24 containing plasmid pANT195 and the control culture *S. lividans* TK24 containing control plasmid pANT849, were visualized by sodium dodecylsulphate polyacrylamide gel electrophoresis. Samples derived from cultures containing plasmid pANT195 revealed a band with M_r of about 42,000, close to the predicted size of daunomycin C-14 hydroxylase. This band was not present in samples derived from the control cultures.

Example B

A 50 ml culture of *S. lividans* TK24 (pANT195) was grown for 48 hours in YEME medium plus 10 μg/ml thiostrepton as in Example 1. This culture was split into 2 X 25ml aliquots, each of which was used to inoculate a 1000 ml flask containing 225 ml of YEME medium plus 10 μg/ml thiostrepton, giving 2 fresh 250 ml cultures, which were grown as described in Example 1 for 48 hours. A 14-liter stirred tank fermentor containing 9.5 liters of YEME medium with 10 μg/ml of thiostrepton was inoculated with both 250 ml cultures, a total inoculum size of 500 ml, and the 10 L culture was incubated for 6 days under the following conditions: temperature, 28°C; air flow, 1 volume air/volume culture/minute; agitation, 250 rpms. The culture was harvested by continuous centrifugation using a Heraeus 300 MD System, from Heraeus Sepatech, South Plainfield, NJ, at 15,000 rpm and a flow rate of 100 ml/min. The resultant pellet was frozen at -70°C until further use. A small portion of the frozen pellet of *S. lividans* TK24 (pANT195) was thawed on ice in ice-cold 0.1 M sodium phosphate (Na_2HPO_4:NaH_2PO_4) buffer having a pH 7.5. The thawed suspension was passed twice through a 4°C French Pressure cell at 15,000 pounds per square inch to break the cells. The broken cell suspension was centrifuged at 10,000 x g for 30 minutes at 4°C and the supernatant from this centrifugation step was kept on ice to provide an isolated partially purified daunomycin C-14 hydroxylase.

P450 Determination

A 100 μl aliquot of the daunomycin C-14 hydroxylase prepared according to Example B, was added to 900 μl of 0.1M sodium phosphate buffer having a pH of 7.5, in a cuvette and approximately 1 mg of sodium dithionite was added to reduce the sample. This sample was used as the background for a spectrophotometric scan from 400 nm to 600 nm. Carbon monoxide was bubbled through this sample for one minute and the sample was scanned again from 400-600 nm. Electronic subtraction of the reduced plus carbon monoxide minus reduced sample revealed a sharp peak at 450 nm, indicative of the active cytochrome P450 enzyme. This assay was used before, during and after Examples 19 to 26 to ensure that the daunomycin C-14 hydroxylase was active and stable. In all cases, the cytochrome P450 activity appeared to be 100% of the original.

Method C

The Fusion Protein

Plasmid pANT199 was introduced by transformation into *E. coli* strain TOP10 from Invitrogen. Transformants were selected using ampicillin and grown in 3.0 ml cultures of SOB medium overnight at 37°C. The recipe for the SOB medium was provided by Invitrogen. Fifty μL of this culture was used to inoculate 3.0 ml of fresh SOB medium. The new culture was grown at 37°C for 2 hours to an optical density of 0.6 and then induced with IPTG at 1.0 mM final concentration for 5 hours. The culture was then harvested by centrifugation in a microcentrifuge and the pellet was frozen overnight at -20°C. The next day the pellet was boiled in SDS-PAGE sample buffer described in Laemmli, U.K. (1970) "Cleavage of Structural Proteins during the assembly of the head of Bacteriophage T4" Nature volume 227, pages 680-685, and run on a 10% (w/v) SDS-PAGE gel. A protein with M_r of about 52,000 was observed that was insert-specific, the approximate size expected for the fusion protein based on amino acid sequence.

The fusion protein is then bound to a nickel-agarose column from Invitrogen, Inc., San Diego, Cal., and washed with 50 mM sodium phosphate buffer at pH 8.0 containing also 300 mM NaCl and 20 mM imidazole. The protein is then eluted using the same buffer but containing with 250 mM imidazole buffer at pH of 8, to provide a pure fusion protein with a modified N-terminus as shown in Figure 9 and SEQ. ID. NO. 8. The leader sequence is then cleaved from the fusion protein using enterokinase available from Biozyme Lab. Int'l Ltd. San Diego Cal., according to the manufacturer's directions, to provide pure N-terminal-modified daunomycin C14 hydroxylase as shown in SEQ. ID. NO. 9.

Methods of Converting Daunomycin to Doxorubicin

A host microorganism transformed with a plasmid containing the *doxA* gene is grown preferably in liquid culture, and daunomycin is added to the culture broth. Preferably, the daunomycin is added at a concentration of from about 2 mg/L to 22.2 mg/L, more preferably about 2 to 10 mg/L. Preferably, the daunomycin concentration is below about 10 mg/L. Where the concentration is above about 10 mg/L, the daunomycin tends to kill the host microorganisms although doxorubicin is still produced. The culture of transformed host microorganism is then incubated with the daunomycin; the longer the incubation the greater the amount of daunomycin is converted to doxorubicin. Preferably, the culture is incubated at least 6 hours, more preferably, at least 24 hours with the daunomycin.

A 48 hour culture has sufficient biomass to convert 2 mg/L daunomycin to doxorubicin within 24 hours.

Next, the doxorubicin is extracted preferably from both the transformed microorganisms and the culture fluid, using conventional techniques. A suitable technique involves extracting the transformed microorganisms and the culture fluid, preadjusted to a pH of about 8.5, with a mixture of chloroform and methanol and separating and drying the organic phase to provide a culture extract. The culture extract is resuspended in methanol and the components of the culture extract are separated, preferably by chromatography, to provide doxorubicin.

Media Composition

GPS production medium contains: glucose, 22.5 g/L; Proflo from Traders, Memphis, TN, 10 g/L; NaCl, 3 g/L; $CaCO_3$, 3 g/L, and 10 ml/L trace salts according to Dekleva, M.L. et. al. (1985), "Nutrient Effects on Anthracycline Production by *Streptomyces peucetius* in a Defined Medium", Canad. J. Microbiol. vol. 31, pages 287-294.

APM seed medium contains the following: yeast extract, 8 g/L; malt extract, 20 g/L; NaCl, 2 g/L 3-(N-morpholino)propanesulfonic acid buffer, 15 g/L; antifoam B from Sigma Chemical Co., St. Louis, MO, 4 ml/L; 10% weight to volume $MgSO_4$, 1 ml/L; 1% weight to volume $FeSO_4$, 1 ml/L; 10% weight to volume $ZnSO_4$, 0.1 ml/L; 50% weight to volume glucose, 120 ml/L, added after autoclaving; tap water to 1.0 L as described in Guilfoile and Hutchinson, (1991), "A Bacterial Analog of the *mdr* Gene of Mammalian Tumor Cells is present in *Streptomyces peucetius*, the Producer of Daunorubicin and Doxorubicin", Proc. Nat'l. Acad. Sci. USA volume 88, pages 8553-8557.

The YEME medium contained 3 g/L yeast extract available from U.S. Biohemical Corp. Cleveland, Ohio; 5 g/L bacto-peptone from Difco Detroit, MI; 3 g/L Difco malt extract; 10 g/L glucose; 200 g/L sucrose; and 2 ml/L of an autoclave-sterilized solution of 2.5 M $MgCl_2 \cdot 6H_2O$. The pH was adjusted to 7.2 and the solution was autoclaved

at 121°C for 20 minutes at 15 psi, to provide the YEME medium. The nitrate-defined-plus-yeast extract medium, also referred to herein as "NDYE medium", contains the following: yeast extract, 5.0 g/L; N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) buffer 4.8 g/L; 0.06 g/L anhydrous $MgSO_4$; 0.24 g/L $K_2HPO_4 \cdot 3H_2O$; 4.28 g/L $NaNO_3$; 1.0 ml/L 20X trace elements; 45% (w/v) glucose solution, 50 ml/L; pH 7.3. The 20X trace elements solution contains the following elements in double distilled water: $ZnCl_2$, 800 mg/L; $FeCl_3 \cdot 6H_2O$, 4000 mg/L; $CuCl_2 \cdot 2H_2O$, 40 mg/L; $MnCl_2 \cdot 4H_2O$, 40 mg/L; $Na_2B_4O_7 \cdot 10H_2O$, 40 mg/L; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 200 mg/L; NiCl, 100 mg/L as described by Dekleva et al. (1985) Can. J. Microbiol volume 31, pages 287-294. A few drops of Mazu DF60-P antifoam, obtained from Mazer Chemical Co., Gurney, IL, were added to control foaming in cultures containing NDYE medium. Other antifoam agents, such as Sigma Antifoam B from Sigma Chemical Company, are also suitable.

EXAMPLES

The following examples are illustrative and not intended to be limiting.

Methods of Producing Doxorubicin Employing Host Microorganisms

Example 1

Cultures of *S. lividans* TK24 containing plasmid pANT195 and control cultures of *S. lividans* TK24 containing plasmid pANT849 were grown on R2YE agar medium containing 10 μg/ml of thiostrepton in standard 100 mm x 15 mm plastic petri dishes for 5 days at 30°C, at which time the entire cultures had sporulated. The spores from one entire petri plate were used to inoculate 50 ml of modified YEME medium in 250 ml erlenmeyer flasks. Then 10 μg/ml thiostrepton in DMSO were added to the medium. The thiostrepton was added as selective pressure to maintain the plasmids. The cultures were grown for 48 hours at 30°C with rotary shaking at 250 rpm, one inch throw on the shaker. After 48 hours, a 1.5 ml sample was removed for plasmid analysis to ensure the presence and size of the insert DNA containing the *doxA* gene using a "mini-prep" procedure according to Carter, M.J., and I.D. Milton, (1993), "An Inexpensive and Simple Method for DNA Purification on Silica Particles," Nucleic Acids Res., Vol. 21, page 1044. Restriction endonuclease digestion with *Bsp*EI and agarose gel electrophoresis generated 1.7 kbp, 2.2 kbp, and 3.14 kbp fragments which indicated an intact plasmid and insert. Then a 10 μL solution containing 100 μg of filter sterilized daun mycin-HCl in distilled water was added to the cultures for a final concentration of 2.0 μg of daunomycin per ml of culture broth. Incubation was continued for 72 hours.

After a total of 120 hours, that is, 48 hours growth and another 72 hours of continued growth in presence of the daunomycin, the pH of the culture was 7.6. The remaining culture broth, a volume of 48.5 ml, was brought to pH 8.5 with the dropwise addition of 5 N NaOH. Then the whole culture broth, including culture fluid and cells, was extracted once with a 2X volume of chloroform:methanol at a ratio of 9:1. The organic phase was separated from the aqueous phase by centrifugation at about 10,000 x g for 10 minutes and then the organic phase was removed by pipet. The organic phase was air dried in a chemical fume hood, then resuspended in 1 ml of 100% reagent grade methanol and spotted onto aluminum-backed, 0.25 mm silica gel thin layer chromatography plates from Whatman, Clifton, NJ. The components derived from the organic phase culture extract were separated using a solvent system of chloroform:methanol:acetic acid:water (80:20:16:6). The anthracyclines in the culture extracts were visualized on the plates by their normal pigmentation and by their fluorescence under ultraviolet irradiation at 365 nm. Table 1 shows the results.

The culture extracts of Example 1 and known standards also were separated and analyzed by high performance liquid chromatography using a C_{18} μBondaPak reverse phase column from Waters Corp. Milford, Ma. The solutions of standards and culture extracts of Example 1 were filtered through 0.2 μm Nylon Acrodisc® 13 filters from Gelman Sciences, Ann Arbor, MI and separated by HPLC using a mobile phase of methanol:water (65:35) brought to a pH of 2.5 with 85% phosphoric acid using a Waters 600E Multisolvent Delivery Pump and Controller and U6K 0-2.0 ml manual injector and detected on-line at 254 nm using a Waters 486 Tunable Absorbance Detector. The data were analyzed on-line and post-run using "Baseline 815" software and a 386 SX PC-compatible computer. The products extracted from the cultures were compared to standards run in parallel and by co-chromatography. These results are shown in Table 2.

TABLE 1

THIN LAYER CHROMATOGRAPHY OF DOXORUBICIN PRODUCED ACCORDING TO EXAMPLE 1 AS COMPARED TO KNOWN STANDARDS

Sample	R_f of Sample
Daunomycin Standard	0.56
Doxorubicin Standard	0.36
13-Dihydrodaunomycin Standard	0.39
Doxorubicin from Cultures Containing Plasmid *doxA* (pANT195)	0.36
13-Dihydrodaunomycin from Control Culture	0.39

A few grains of each standard was reconstituted in 1 ml of methanol.

As indicated by the results in Table 1, th cultures transformed with plasmid pANT195 containing *doxA* which were incubated with

daunomycin, produced doxorubicin. In contrast, the control cultures produced 13-dihydrodaunomycin. Co-chromatography confirmed these results.

TABLE 2

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF DOXORUBICIN PRODUCED ACCORDING TO EXAMPLE 1 AS COMPARED TO KNOWN STANDARDS

Sample	Retention time of sample[2] (minutes)
Daunomycin[1] Standard	13.3
Doxorubicin[1] Standard	8.4
13-Dihydrodaunomycin Standard	10.6
Doxorubicin from *doxA* Transformants	4.1; 7.2 (minor) 8.4 (major)
Control	4.1; 10.6; 21.2

[1] Standards were reconstituted at 1.0 mg/ml in methanol.
[2] A methanol peak at 2.9 minutes was found in all samples.

The *doxA* transformants converted greater than 90% of the daunomycin to doxorubicin in 72 hours as evidenced by both TLC and HPLC analyses. The control cultures, which lack the *doxA* gene, converted daunomycin to 13-dihydrodaunomycin, but not to doxorubicin.

The culture extracts of Example 1 and the standards were hydrolyzed to their respective aglycones to verify the chemical structures. The acid hydrolysis product of the doxorubicin is adriamycinone, and the acid hydrolysis product of the 13-dihydrodaunomycin is 13-dihydrodaunomycinone. The acid hydrolysis product of the doxorubicin produced by Example 1 was adriamycinone. The acid hydrolysis product of the 13-dihydrodaunomycin produced by control cultures of Example 1 was 13-dihydrodaunomycinone.

Example 2

The procedure of Example 1 was repeated, with the following exceptions. The cultures were grown at 28°C rather than 30°C, for 48 hours. After 48 hours of growth, 500 μg of daunomycin-HCl were added to the cultures for a final concentration of 10.0 μg/ml of daunomycin followed by further incubation for 36 hours, instead of 72 hours.

The culture broth then was extracted and the entire sample volume was spotted in a line onto a 250 μm layer thickness 20 cm x 20 cm glass-backed TLC plate containing a fluorescent indicator (254 nm) from Aldrich, Milwaukee, WI. The doxorubicin was separated from contaminants by chromatography for 2 hours using a mobile phase of chloroform:methanol:acetic acid:water (80:20:16:6), after which the silica gel containing the band having R_f, 0.3 - 0.4 was scraped from the

plate. The silica gel was extracted three times with about 1.5 ml of methanol each time. The methanol extracts were combined, filtered through a 0.2 μm Nylon Acrodisc® 13 filter, and air-dried. The dried product was resuspended in 500 μL of chloroform:methanol in a ratio of 9:1, back-extracted with an equal volume of water that had been previously made alkaline to pH 10.0 using Na_2CO_3, and the organic phase from this extraction procedure was removed and dried. The dried sample was resuspended in 500 μL of methanol, from which 50 μL were removed for HPLC and TLC analysis. HPLC analysis of this sample confirmed that *doxA* transformant cultures converted virtually all of the 500 μg of daunomycin to doxorubicin.

The remainder was redried and subjected for mass spectrometry analysis. MS spectra were recorded on a SCIEX API III+ triple quadruple mass spectrometer fitted with an atmospheric pressure chemical ionization source operating in a positive ion mode. MS spectra were acquired by scanning the first quadruple (Q1), the results are shown in Table 3.

TABLE 3

MS ANALYSIS ON THE DOXORUBICIN PRODUCED ACCORDING TO EXAMPLE 2

Sample	Calculated MW	Average M+1
Daunomycin standard	527.51	528.00
Doxorubicin standard	543.54	544.05
13-Dihydrodaunomycin standard	529.50	530.01
Doxorubicin from Transformants Containing *doxA*	----	544.00

The results of the MS analysis, shown in Table 3, indicate that the doxorubicin from *doxA* transformed cultures has an M+1 of 544.00, essentially the same value as obtained with the doxorubicin standard. The M+1 value of the doxorubicin produced by the *doxA* transformed culture was not similar to the M+1 values obtained with either daunomycin standard or the 13-dihydrodaunomycin standard.

Example 3

A 50 ml culture of *Streptomyces lividans* TK24(pANT195) culture was prepared as in Example 1. This culture was grown for 48 hours at 28°C and then 25 ml were removed and used to inoculate 200 ml of YEME medium containing 10 μg/L of thiostrepton in a 1.0 L flask, having total, 225 ml of culture volume. After incubation for 48 hours at 28°C, 5.0 mg of daunomycin-HCl in 1000 μl of distilled water were added to the culture to give a final concentration of 22.2 μg/ml. A control culture of *S. lividans* TK24 containing plasmid pANT849 which lacks the *doxA* gene, was incubated in the presence of 100 μg of daunomycin-HCl. The cultures were incubated for 48 hours and then extracted as described in Example 1 and analyzed by HPLC and TLC. The doxorubicin which migrated in a broad band having an R_f of 0.3 - 0.4 was separated from contaminants by chromatography and prepared for MS analysis as in Example 2. The results are shown in Table 4.

The *doxA* transformed culture converted essentially all of the 5 mg of daunomycin to doxorubicin. Notably, the *doxA* transformed culture was virtually dead at the end of 48 hours, whereas the cultures of Examples 1 and 2 which received 2 μg/ml of daunomycin were fully viable. Nevertheless, even though the culture was eventually killed by the daunomycin, the culture converted essentially all of the daunomycin to doxorubicin.

HPLC analysis showed that the *doxA* transformed cultures converted greater than 95% of the daunomycin to doxorubicin. The control culture converted essentially 100% of the daunomycin to 13-dihydrodaunomycin, and did not produce doxorubicin.

TABLE 4

MASS SPECTROPHOTOMETRY ANALYSIS ON THE DOXORUBICIN PRODUCED ACCORDING TO EXAMPLE 3

Sample	Calculated MW	Average M+1
Doxorubicin Standard	543.54	543.65
13-Dihydrodaunomycin Standard	529.50	529.85
Doxorubicin from Transfomants Containing *doxA*	----	543.90
13-Dihydrodaunomycin from Control culture	----	529.15

The results of the MS analysis, shown in Table 4, indicate that the doxorubicin produced by cultures containing plasmid pANT195, has an M+1 of 543.90, essentially the same as obtained with doxorubicin standard. An MS-MS analysis was run on the parent 543.9 peak and is shown in Table 5.

TABLE 5

MASS SPECTROPHOTOMETRY ANALYSIS OF DOXORUBICIN PRODUCED ACCORDING TO EXAMPLE 3

Sample	M+1	Major fragmentation
Doxorubicin Standard	543.65	489.90[m], 396.95, 378.70, 360.45, 345.95[m], 320.85, 299.45, 130.15
Doxorubicin produced according to Example 3	543.90	396.80, 378.90, 361.00, 130.20
13-Dihydrodaunomycin Standard	529.85	382.85, 365.30, 346.35, 320.85, 129.35, 113.10
Control culture extract	529.15	497.20[m], 482.00[m], 382.80, 364.60, 320.75, 305.75, 129.95

[1] The product sample was significantly less concentrated than the standard sample, leading to recovery of only the most abundant fragmentation species. [m] - minor fragmentation species.

The MS-MS analysis on 543.90 peak from the doxorubicin produced in Example 3 shows daughter peaks which are essentially identical to the daughter peaks from the doxorubicin standard. Thus, the doxorubicin produced by the transformants containing *doxA* has the M+1 and MS/MS fragmentation patterns of standard doxorubicin. Similarly, the culture extract from the control culture had an M+1 and fragmentation pattern similar to that of standard 13-dihydrodaunomycin.

Example 4

Fifty ml cultures of *Streptomyces lividans* TK24(pANT195) and *S. lividans* TK24(pANT849) as a control were inoculated and prepared as in Example 1 except that they were grown at 28°C for 48 hours. At that time, 100 μg of daunomycin in 10 μL of distilled water was added to the cultures for a final concentration of 2 μg/ml. The cultures were further incubated for 24 hours. The cultures were then extracted as described in Example 1 and subjected to HPLC analysis.

The transformed culture containing the *doxA* gene converted greater than 95% of the daunomycin to doxorubicin within 24 hours. The control culture converted approximately 100% of the daunomycin to 13-dihydrodaunomycin.

Example 5

The procedure of Example 4 was repeated except that 100 μg of 13-dihydrodaunomycin, rather than daunomycin, were added to the cultures and the cultures were further incubated for 48 hours rather than 24 hours.

In 48 hours, the culture containing plasmid *doxA* (pANT195) converted 100% of the 13-dihydrodaunomycin to doxorubicin. The control culture did not convert the 13-dihydrodaunomycin.

Example 6

Fifty ml cultures of *Streptomyces lividans* TK24(pANT196) and *S. lividans* TK24(pIJ702) as a control, were treated as in Example 4 except that the cultures were incubated with daunomycin for 72 hours.

The culture containing plasmid pANT196, which contains the *doxA* gene expressed from the *melC1* promoter, converted 20% of the daunomycin to doxorubicin. Thus the *melC1* promoter is less preferred than the snpA promoter. The control cultures converted 100% of the daunomycin to 13-dihydrodaunomycin.

Example 7

50 ml cultures of *Streptomyces lividans* TK24(pANT192), having wild type and *snpA* promoters, *Streptomyces lividans* TK24(pANT193), having wild type and *snpA* promoters, *Streptomyces lividans* TK24(pANT194) which lacks the *snpA*-promoter and *snpR* activator gene, and *S. lividans* TK24(pANT849) as a control, were prepared and analyzed prepared as in Example 4, except that the cultures were incubated in the presence of the daunomycin for 48 hours. The results are presented in Table 6.

TABLE 6

COMPARISON OF DIFFERENT PLASMIDS ON PERCENT CONVERSION OF DAUNOMYCIN TO DOXORUBICIN

Plasmid	Products
pANT192	75% 13-dihydrodaunomycin/25% doxorubicin
pANT193	80% doxorubicin/20% 13-dihydrodaunomycin
pANT194	90% 13-dihydrodaunomycin/ 10% doxorubicin
pANT195	100% doxorubicin
pANT849 (control)	100% 13-DHD

As shown in Table 6, the culture containing pANT192, which contains *doxA*, converted 25% of daunomycin to doxorubicin and 75% of daunomycin to the 13-dihydrodaunomycin. The culture containing plasmid pANT193, which contains *doxA*, converted 80% of daunomycin to doxorubicin and 20% of daunomycin to the 13-dihydrodaunomycin. The culture containing plasmid pANT194, which contains *doxA* but lacks the *snpA*-promoter and *snpR* activator, converted 10% of daunomycin to doxorubicin and 90% of daunomycin to the 13-dihydrodaunomycin. The

control culture converted the daunomycin to 13-dihydrodaunomycin, but not doxorubicin.

Example 8

Fifty ml cultures of *Streptomyces lividans* TK24(pANT195) and *S. lividans* TK24(pANT849) as a control, were prepared as in Example 4, except that the pH of the YEME medium was adjusted before inoculation using NaOH or HCl to provide an initial culture pH as shown in Table 7. The cultures were further incubated for 48 hours, rather than 24 hours. The results are shown in Table 7.

TABLE 7

EFFECT OF CULTURE PH ON DOXORUBICIN PRODUCTION

Initial pH	Final pH	Percent Daunomycin bioconverted to Doxorubicin
6.0	7.0	50%
6.5	7.6	90%
7.0	7.9	100%
7.5	7.0	100%
8.0	---	No growth or bioconversion

The % conversion is approximate.

The culture containing plasmid pANT195 which contains *doxA*, and which was initially at pH of 7.0 or 7.5, converted 100% of the daunomycin to doxorubicin. The cultures containing plasmid *doxA* which were initially at pH 6.0 converted 50% of the daunomycin to doxorubicin. The cultures containing plasmid *doxA* which were initially at pH 6.5 converted 90% of the daunomycin to doxorubicn. Accordingly it is preferred that the transformed host cultures be grown at an initial pH of higher than 6.5.

Example 9

The procedure of Example 4 was repeated except that cultures were grown at either 22°C, 28°C, or 37°C and incubated with the daunomycin for 48 hours at such temperatures.

All three of the cultures containing plasmid *doxA* converted 100% of the daunomycin to doxorubicin.

Example 10

A 50 ml culture of *Streptomyces lividans* TK24(pANT195), inoculated and prepared as in Example 1, was grown at 28°C for 48 hours. At that time, the cultures were harvested by centrifugation at 10,000 x *g* in a high speed centrifuge, washed once with 100 mM 3-(N-morpholino)propanesulfonic acid buffer at pH 7.2. The cells from the cultures were reconstituted in 5.0 ml of the 100 mM 3-(N-

morpholino)propanesulfonic acid buffer to give a final volume of 6.0 ml which included the volume of the packed cell mass, resulting in an approximately 8-fold concentration of the recombinant mycelia in buffer. Then 100 μg of daunomycin were added in 10 μL of distilled water for a final concentration of 16.7 μg/ml of daunomycin. A concentration of 16.7 μg/ml of daunomycin is toxic to the host. The culture was further incubated for 7.0 hours, after which it was extracted as described in Example 1 and subjected to HPLC analysis.

In 7 hours, the concentrated cultures containing the plasmid with a *doxA* insert converted about 25% of the daunomycin to doxorubicin.

Example 11

Five ml each of APM seed medium containing 10 μg/ml of thiostrepton were inoculated by loop from R2YE agar plates, containing 50 μg/ml thiostrepton of *S. peucetius* 29050(pANT195) and *S. peucetius* 29050(pANT849). Each culture was grown at 28°C for 48 hours. Fifty ml each of GPS "production" medium containing 10 μg/ml of thiostrepton were inoculated with 2.5 mls of seed culture grown in APM seed medium. The cultures were grown at 28°C for 48 hours as in Example 1. Then 100 μg of daunomycin in 10 μL of distilled water for a final concentration of 2 μg/ml of daunomycin was added to the cultures. The cultures were further incubated for 48 hours, then extracted as described in Example 1.

After 48 hours, the culture containing plasmid pANT195, which contains the *doxA* gene, converted about one-half of the daunomycin to doxorubicin. The control cultures did not convert daunomycin to doxorubicin.

Example 12

Fifty ml cultures of *Streptomyces coelicolor* CH999 (pANT195), and *Streptomyces coelicolor* CH999 (pANT849) as a control, were used in the procedure of Example 4 except that the cultures were incubated with the daunomycin for 48 hours.

Again the cultures containing pANT195 converted 100% of the daunomycin to doxorubicin, while the control converted 80% of the daunomycin to 13-dihydrodaunomycin. In the control cultures 20% of the daunomycin was not converted.

Example 13

The procedure of Example 12 was repeated, except that after the daunomycin addition, the cultures were only incubated for 1, 2, or 4 hours.

After 1 hour, 0.7% of the daunomycin was converted to doxorubicin by the cultures which contained pANT195. After 2 hours, 1.0% of the daunomycin was converted and by 4 hours 15% of the daunomycin was converted to doxorubicin. The control cultures which

lacked the plasmid containing *doxA* did not convert any of the daunomycin.

Example 14

50 ml cultures of *Streptomyces* sp. strain C5(pANT195) and *Streptomyces* sp. strain C5(pANT849) as a control, were grown at 28°C for 72 hours in NDYE medium as described in Example 1. After 72 hours, 100 μg of unlabelled daunomycin and 5 μCi of ^{3}H-daunomycin, having a specific radioactivity of 5.0 Ci/mmol, were added to each culture and they were incubated for another 48 hours. The cultures were extracted as described in Example 1 and analyzed by TLC and autoradiography.

The cultures of containing plasmid pANT195 converted approximately 5% of the radiolabelled daunomycin to doxorubicin. No other products other than the substrate daunomycin and doxorubicin were observed in these cultures. The control cultures converted approximately 90% of the radiolabelled daunomycin to baumycin A1 and baumycin A2.

Example 15

50 ml cultures of *Streptomyces* sp. strain C5, which does not appear to synthesize doxorubicin, and the following mutants of *Streptomyces* sp. strain C5: SC5-*dauA74*, SC5-*dauCE147*, SC5-*dauE24*, and SC5-*dauH54*, were grown for 48 hours in NDYE medium. These mutants do not synthesize daunomycin. At 48 hours, 100 μg of daunomycin was added for a final concentration of 2 μg/ml, and the cultures were incubated for 36 hours. The products were extracted as in Example 1 and subjected to HPLC analysis.

All *Streptomyces* sp. strain C5 cultures, each of which lacked a plasmid containing *doxA*, converted about 10% of the daunomycin to doxorubicin. Baumycins A1 and A2 were also detected.

Example 16

The procedure of Example 4 was repeated except that the cultures were then incubated for 48 hours with 100 μg, for a final concentration of 2ug/ml of one of the following: carminomycin, idarubicin, daunomycinone, or carminomycinone.

Incubation of *S. lividans* TK24(pANT195) cultures with carminomycin or idarubicin, resulted in greater than 85% recovery of carminomycin and idarubicin. The cultures which contained plasmid pANT195 converted 100% of the daunomycinone to 13-hydroxydaunomycinone and 100% of the carminomycinone to 13-hydroxycarminomycinone. The control cultures, which lack the *doxA* gene, converted 100% of the carminomycin, idarubicin, daunomycinone, and carminomycinone to their 13-dihydro derivatives.

Example 17

The procedure of Example 4 was repeated except that the cultures received 100 μg of 13-dihydrocarminomycin rather than daunomycin. The

cultures were then incubated for 36 hours and analyzed by TLC and HPLC.

The cultures containing plasmid pANT195 converted 100% of the 13-dihydrocarminomycin to carminomycin. No other products were observed. In the control culture, none of the 13-dihydrocarminomycin was converted. The *doxA* gene confers the ability to oxidize the C-13 hydroxyl function of the 13-dihydro-carminomycin to a keto function.

Novel Synthesis of 13-deoxycarminomycin and 13-deoxydaunomycin

Example 18

ε-Rhodomycin D compound was converted to both 13-deoxycarminomycin and 13-deoxydaunomycin, by host microorganisms containing plasmid which contains the *Streptomyces sp.* strain C5 *dauP* gene which encodes ε-rhodomycin D esterase and the *Streptomyces sp.* strain C5 *dauK* gene which encodes carminomycin 4-O-methyltransferase. Plasmid pANT144 as shown in Figure 12, and described in Dickens, M.L., et. al., (1995) "Analysis of Clustered Genes Encoding both Early and Late Steps in Daunomycin Biosynthesis by *Streptomyces sp.* strain C5" J. Bacteriol. volume 177, pages 536-543, was introduced into *S. lividans* TK24 by protoplast transformation. *S. lividans* TK24 (pANT144) was grown for 48 hours in 50 ml of YEME medium containing 10 μg/ml of thiostrepton and then used to inoculate 450 ml of the YEME medium which contained 10 μg/ml of thiostrepton for a total culture volume of 500 ml, in a two liter flask. The resultant 500 ml culture was incubated for 48 hours at 28°C as in Example 1. Next, 5.0 mg of ε-rhodomycin D, the glycone of ε-rhodomycinone, from the National Cancer Institute, Drug Synthesis and Chemistry Branch, Bethesda, MD designated compound #263854-H, were added to the culture for a final concentration of 10 μg/ml and the cultures were incubated for an additional 48 hours. The culture then was adjusted to pH 8.5, and then extracted twice, each with 1 volume of chloroform:methanol (9:1) as in Example 3. The organic extract was reduced to dryness, reconstituted in 500 μL of chloroform:methanol (9:1), filtered, back extracted, dried, and reconstituted in 2.0 ml of methanol. The extract was separated and extracted as in Example 2. R_f values for 13-deoxycarminomycin and 13-deoxydaunomycin were approximately 0.60 and 0.64, respectively. The 13-deoxycarminomycin and 13-deoxydaunomycin were reduced to dryness and each was brought up again in 50 μl of methanol.

Methods f Producing Anthracyclines Employing Daunomycin C-14 Hydroxylas

Example 19

904 μl of the daunomycin C-14 hydroxylase produced according to the method of Example B, containing approximately 1 mg of total

protein, was incubated in a 16 mm well of a 24 well culture plate at 30°C with shaking for 2 hours with 25 μg in 5 μl of either daunomycin, 13-dihydrodaunomycin, or 13-dihydrocarminomycin. The final volume of each well was 1.0 ml; 0.1M sodium phosphate buffer at pH 7.5, was added to bring the total volume to 1 ml, as needed. After 2 hours of incubation, the pH of each reaction mixture was increased to pH 8.5 using 1 M NaOH and each was extracted twice each with 500 μl of chloroform: methanol (9:1). The organic layers were combined, reduced to dryness, reconstituted in 10 μl of methanol, separated and analyzed by TLC as in Example 1.

The daunomycin C-14 hydroxylase converted 50% of the 13-dihydrocarminomycin to carminomycin and 50% of the 13-dihydrodaunomycin to daunomycin.

Example 20

The procedure of example 19 was repeated except that 10 μl of NADH, 1 mM final concentration; 10 μl of NADPH, 1 mM final concentration, were added.

The daunomycin C-14 hydroxylase converted 100% of the 13-dihydrocarminomycin to carminomycin and 100% of the 13-dihydrodaunomycin to daunomycin.

Example 21

The procedure of Example 20 was repeated except that the following reagents, available from Sigma Chemical Co., were added: 20 μl of glucose-6-phosphate; 10 mM final concentration; 10 μl of NADP$^+$, 1 mM final concentration; 1.0 μl of glucose-6-phosphate dehydrogenase; 0.84 units, final activity; 20 μl of spinach ferredoxin, 44 μg final concentration; and 10 μl of spinach ferredoxin-NADP$^+$ reductase, 0.05 units final activity. The glucose-6-phosphate, glucose-6-phosphate dehydrogenase and NADP$^+$ constitute a "NADPH-regenerating system". After 2 hours of incubation, the extracts were extracted and analyzed by TLC and HPLC.

The daunomycin C-14 hydroxylase converted 100% of the 13-dihydrocarminomycin to carminomycin and 100% of the 13-dihydrodaunomycin to daunomycin, as shown by TLC. HPLC revealed the conversion of about 5% of the daunomycin to doxorubicin.

Example 22

The procedure of Example 21 was repeated, except 10 μl of flavin adenine mononucleotide from Sigma, 10 μg final concentration; and 10 μg in 10 μl, flavin adenine dinucleotide, were also added.

100% of the 13-dihydrocarminomycin was converted to carminomycin and 100% of the 13-dihydrodaunomycin was converted to daunomycin by the daunomycin C-14 hydroxylase. Doxorubicin was not detected using TLC.

Example 23

The procedure of Example 20 was repeated except that different anthracyclines were used, incubation was for 1 hour and 10 μl of NADP+, 1 mM final concentration was added. 25 μg in 5 μl the following anthracyclines were used: either ε-rhodomycin D, 13-deoxydaunomycin from example 18, or 13-deoxycarminomycin, from example 18.

About 20% of the 13-deoxycarminomycin was converted to 13-dihydrocarminomycin and about 80% was converted to carminomycin; and 13-deoxydaunomycin was converted to about 20% 13-dihydrodaunomycin and about 80% daunomycin. The ε-rhodomycin D did not appear to be converted.

Thus, the daunomycin C-14 hydroxylase converted 13-deoxycarminomycin to 13-dihydrocarminomycin and carminomycin; 13-dihydrocarminomycin to carminomycin; 13-deoxydaunomycin to 13-dihyrodaunomycin and daunomycin; and 13-dihydrodaunomycin to daunomycin. Thus, daunomycin C-14 hydroxylase catalyzes the oxidation of the C-13 methylene to a C-13 hydroxyl function, and catalyzes the oxidation of the C-13 hydroxyl function to C-13 keto function. The daunomycin C-14 hydroxylase is useful for making 13-dihydrocarminomycin, carminomycin, 13-dihydrodaunomycin and daunomycin.

Example 24

The procedure of Example 22 was repeated except that the daunomycin C-14 hydroxylase was incubated with daunomycin for 18 hours rather than 2 hours and in 25 ml erylenmyer flasks shaken at 250 rpm on a rotary shaker.

Doxorubicin was not detected by HPLC or TLC.

Example 25

The procedure of Example 24 was repeated except that the reagent volumes were tripled. The reagent concentrations however were not increased.

Approximately 5% of the daunomycin was converted to doxorubicin as determined by HPLC.

Example 26

The procedure of Example 24 was repeated except that the reagent volumes were quintupled. The reagent concentrations however, were not increased.

Approximately 20 to 25% of the daunomycin was converted to doxorubicin, as determined by HPLC.

The present invention includes: the DNA sequences encoding a protein daunomycin C-14 hydroxylase, which adds a hydroxyl group to carbon 14 of daunomycin; the messenger RNA transcript of such DNA sequence; and an isolated protein which adds a hydroxyl group to carbon 14 of daunomycin.

For example, the DNA sequences include: DNA molecules which, but for the degeneracy of the genetic code would hybridize to DNA encoding the daunomycin C-14 hydroxylase, thus the degenerate DNA which encodes the daunomycin C-14 hydroxylase protein; DNA strands complementary to: DNA sequences encoding the daunomycin C-14 hydroxylase protein including DNA in Figures 3, 6, 9 and 11; heterologous DNA having substantial sequence homology to the DNA encoding the daunomycin C-14 hydroxylase protein, including the DNA sequences in Figures 3, 6, 9 and 11 or portions thereof.

The daunomycin C-14 hydroxylase protein includes, for example, the daunomycin C-14 hydroxylase protein of strains other than *Streptomyces* sp. strain C5; proteins having 75% homology to the proteins in Figures 3, 6, 9 and 11, and proteins or portions thereof having substantially the same amino acid sequence as shown in Figures 3, 6, 9 and 11.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Dickens, Michael L.
DeSanti, Charles L.

(ii) TITLE OF INVENTION: METHOD OF PRODUCING DOXORUBICIN

(iii) NUMBER OF SEQUENCES: 9

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GACATGCATG CGGAGGGGTG CCTC
24

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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48

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3196 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 1498..2764

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1498..2764

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGTACCCGCG CATCGATGTC ATGGCCGGCA ACGCCGGCGG CATGTTCTGG TCGCGCACCA 60

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CACGGCTGCT GCGGGAGCGG CTCGCGGGCG GGCGGTTGAT CCTCACCTCG TCCGACGCGT 180

ACACCCAGGG CCGGATCGAC CCGGACGATC TCAACGGCGA CCGTCACCGC TACAGCGCGG 240

GCCAGGCGTA CGGCACGTCC AAACAGGCCA ACATCATGAC CGCCACGGAG GCCGCCCGGC 300

GCTGGCCGGA CGTGCTGACG GTCAGCTACC ACCCCGGCGA GGTCCGCACC CGCATCGGGC 360

GGGGCACAGT CGCCTCGACC TACTTCCGGT TCAACCCCTT CCTGCGGTCC GCGGCCAAGG 420

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GCGCCGACAC TCTCGTGTGG CTGGCGGCCG CGCCGGCCGA GGAGTTGACC ACGGGCGGCT      480

ACTACAGCGA CCGGCGGCTG TCCCCGGTGA GCGGCCCGAC CGCCGACGCC GGCCTCGCGG      540

CCAAGCTCTG GGAGGCCAGC GCGGCCGCCG TCGGCCACAC CGCGCGCTGA CCGCGGCGGG      600

CCTCCCCGCC CGCATGCCCG TCTCATCCGC GAGCGCAGAC GCTCGTGTGC CGATCCGTCG      660

AAAGGAACGA TTCGTGACCA GGTTCGCGCC CGGCGCCCCC GCATGGTTCG ACCTCGGGTC      720

GCCCGATGTC GCCGCCTCGG CCGACTTCTA CACCGGCCTC TTCGCGTGGA CCGCGACCGT      780

GGTCAGCGAC CCGGGTGCCG GGGGATACAC TACTTTCAGC TCCGACGGGA AGCCTGTCGC      840

CGCGGTCGCC CGCCATCAGA TCGACACGCC CTACCACCGT CCGTACGGGC CCGGCAAGCA      900

CCAGCACGGC ATGCCGGCCA TCTGGACCGT GTACTTCGCC ACCAACGACG CCGACGCACT      960

GACCAAACGG GTCGAAGCGG CGGGTGGCGA CGTCATCATG CACCCGATGG ACGTCCTCGG     1020

TCTCGGCCGG ATGGCGGTCT TCGCCGACCC ATCGGGGGCC GCGTTCGCGG TGTGGCGCAA     1080

GGGCGTCATG GAGGGCGCGG AGGTGACGGG CGTGCCCGGC TCGGTCGGCT GGGTGGAACT     1140

GGTGACCGAC GACATCGGGA CCGCCCGTGG CTTCTACCGT GCGACCCTCG GCCTGGCTCC     1200

GGCCGACACC GGACGCAAGG GCGTCACCGA CCCGGTTTGG CACATCCATG ACACACCGGT     1260

CGCCGGCACC CGGGAACTGG GCACGACCGG CGCGGTACGG CCCCACTGGG CCGTGCTGTT     1320

CTCCGTGCAC GACTGCGACG CGACGGTCCG GCGGGCCGTC GAACTCGGCG GCTCCGTCGA     1380

GAACGAGCCC GTCGACACCC CCAGGGGGCG GCGGGCGGAC CTGCTCGACC CGCACGGGGC     1440

CGGCTTCTCG GTGGTCGAAC TGCGGGAGGC GTACCCCGCG GCGGCGGACG GTGCCTC        1497

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 1               5                  10                  15

ATG ATG ACC ATG CAG CGC AAA CCC GAG GTG CAC GAC GCA TTC CGA GAG        1593
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            20                  25                  30

GCG GGC CCC GTC GTC GAG GTG AAC GCC CCC GCG GGC GGA CCC GCC TGG        1641
Ala Gly Pro Val Val Glu Val Asn Ala Pro Ala Gly Gly Pro Ala Trp
        35                  40                  45

GTC ATC ACC GAT GAC GCC CTC GCC CGC GAG GTG CTG GCC GAT CCC CGG        1689
Val Ile Thr Asp Asp Ala Leu Ala Arg Glu Val Leu Ala Asp Pro Arg
    50                  55                  60

TTC GTG AAG GGA CCC GAT CTC GCG CCC ACC GCC TGG CGG GGG GTG GAC        1737
Phe Val Lys Gly Pro Asp Leu Ala Pro Thr Ala Trp Arg Gly Val Asp
65                  70                  75                  80

GAC GGT CTC GAC ATC CCC GTT CCG GAG CTG CGT CCG TTC ACG CTC ATC        1785
Asp Gly Leu Asp Ile Pro Val Pro Glu Leu Arg Pro Phe Thr Leu Ile
                85                  90                  95

GCC GTG GAC GGT GAG GAC CAC CGG CGT CTG CGC CGC ATC CAC GCA CCG        1833
Ala Val Asp Gly Glu Asp His Arg Arg Leu Arg Arg Ile His Ala Pro
            100                 105                 110

GCG TTC AAC CCG CGC CGG CTG GCC GAG CGG ACG GAT CGC ATC GCC GCC        1881
Ala Phe Asn Pro Arg Arg Leu Ala Glu Arg Thr Asp Arg Ile Ala Ala
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        115                 120                 125

ATC GCC GAC CGG CTG CTC ACC GAA CTC GCC GAC TCC TCC GAC CGG TCG      1929
Ile Ala Asp Arg Leu Leu Thr Glu Leu Ala Asp Ser Ser Asp Arg Ser
    130                 135                 140

GGC GAA CCG GCC GAG CTG ATC GGC GGC TTC GCG TAC CAC TTC CCG CTG      1977
Gly Glu Pro Ala Glu Leu Ile Gly Gly Phe Ala Tyr His Phe Pro Leu
145                 150                 155                 160

TTG GTC ATC TGC GAA CTG CTC GGC GTG CCG GTC ACC GAT CCG GCA ATG      2025
Leu Val Ile Cys Glu Leu Leu Gly Val Pro Val Thr Asp Pro Ala Met
                165                 170                 175

GCC CGC GAG GCC GTC GGC GTG CTC AAG GCA CTC GGC CTC GGC GGC CCG      2073
Ala Arg Glu Ala Val Gly Val Leu Lys Ala Leu Gly Leu Gly Gly Pro
            180                 185                 190

CAG AGC GCC GGC GGT GAC GGC ACG GAC CCT GCC GGG GAC GTG CCG GAC      2121
Gln Ser Ala Gly Gly Asp Gly Thr Asp Pro Ala Gly Asp Val Pro Asp
        195                 200                 205

ACG TCG GCG CTG GAG AGC CTT CTC CTC GAA GCC GTG CAC GCG GCC CGG      2169
Thr Ser Ala Leu Glu Ser Leu Leu Leu Glu Ala Val His Ala Ala Arg
    210                 215                 220

CGG AAA GAC ACC CGG ACC ATG ACC CGC GTG CTC TAT GAA CGC GCA CAG      2217
Arg Lys Asp Thr Arg Thr Met Thr Arg Val Leu Tyr Glu Arg Ala Gln
225                 230                 235                 240

GCA GAG TTC GGC TCG GTC TCC GAC GAC CAG CTC GTC TAC ATG ATC ACC      2265
Ala Glu Phe Gly Ser Val Ser Asp Asp Gln Leu Val Tyr Met Ile Thr
                245                 250                 255

GGA CTC ATC TTC GCC GGC CAC GAC ACC ACC GGC TCG TTC CTG GGC TTC      2313
Gly Leu Ile Phe Ala Gly His Asp Thr Thr Gly Ser Phe Leu Gly Phe
            260                 265                 270

CTG CTT GCG GAG GTC CTG GCG GGC CGT CTC GCG GCG GAC GCC GAC GGG      2361
Leu Leu Ala Glu Val Leu Ala Gly Arg Leu Ala Ala Asp Ala Asp Gly
        275                 280                 285

GAC GCC ATC TCC CGG TTC GTG GAG GAG GCG CTG CGC CAC CAC CCG CCG      2409
Asp Ala Ile Ser Arg Phe Val Glu Glu Ala Leu Arg His His Pro Pro
    290                 295                 300

GTG CCC TAC TCG TTG TGG AGG TTC GCT GCC ACG GAG GTG GTC ATC CGC      2457
Val Pro Tyr Ser Leu Trp Arg Phe Ala Ala Thr Glu Val Val Ile Arg
305                 310                 315                 320

GGT GTC CGG CTG CCC CGC GGA GCG CCG GTA CTG GTG GAC ATC GAG GGC      2505
Gly Val Arg Leu Pro Arg Gly Ala Pro Val Leu Val Asp Ile Glu Gly
                325                 330                 335

ACC AAC ACC GAC GGC CGC CAT CAC GAC GCC CCG CAC GCT TTC CAC CCG      2553
Thr Asn Thr Asp Gly Arg His His Asp Ala Pro His Ala Phe His Pro
            340                 345                 350

GAC CGC CCT TCG AGG CGG CGG CTC ACC TTC GGC GAC GGG CCG CAC TAC      2601
Asp Arg Pro Ser Arg Arg Arg Leu Thr Phe Gly Asp Gly Pro His Tyr
        355                 360                 365

TGC ATC GGG GAG CAG CTC GCC CAG CTG GAA TCG CGC ACG ATG ATC GGC      2649
Cys Ile Gly Glu Gln Leu Ala Gln Leu Glu Ser Arg Thr Met Ile Gly
    370                 375                 380
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GTA CTG CGC AGC AGG TTC CCC CAA GCC CGA CTG GCC GTG CCG TAC GAG      2697
Val Leu Arg Ser Arg Phe Pro Gln Ala Arg Leu Ala Val Pro Tyr Glu
385                 390                 395                 400

GAG TTG CGG TGG TGC AGG AAG GGG GCC CAG ACA GCG CGG CTC ACT GAC      2745
Glu Leu Arg Trp Cys Arg Lys Gly Ala Gln Thr Ala Arg Leu Thr Asp
                405                 410                 415

CTG CCC GTC TGG CTG CGT T GATGGGCCGA CCGCGACCCG GCACGGGACC            2794
Leu Pro Val Trp Leu Arg
            420

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GCCATGCACA CTTTCATGGC GGACATTCAC TTGCGAGGAT GGAGTGAGCA CACGGGGCCG    2974

CCCGAGACAC CCTACGGCCG CCGGAAGTAT GCCACCTGTT GACGCGAATG GAACGCCACA    3034

GAGGGAGCAC CGGCAATGCA GATCAATATG TTGGGCCCGC TCGTTGCACA TCACAATGGC    3094

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 422 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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            20                  25                  30

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    50                  55                  60

Phe Val Lys Gly Pro Asp Leu Ala Pro Thr Ala Trp Arg Gly Val Asp
65                  70                  75                  80

Asp Gly Leu Asp Ile Pro Val Pro Glu Leu Arg Pro Phe Thr Leu Ile
                85                  90                  95

Ala Val Asp Gly Glu Asp His Arg Arg Leu Arg Arg Ile His Ala Pro
            100                 105                 110

Ala Phe Asn Pro Arg Arg Leu Ala Glu Arg Thr Asp Arg Ile Ala Ala
        115                 120                 125

Ile Ala Asp Arg Leu Leu Thr Glu Leu Ala Asp Ser Ser Asp Arg Ser
    130                 135                 140

Gly Glu Pro Ala Glu Leu Ile Gly Gly Phe Ala Tyr His Phe Pro Leu
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145 150 155 160

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195 200 205

Thr Ser Ala Leu Glu Ser Leu Leu Leu Glu Ala Val His Ala Ala Arg
210 215 220

Arg Lys Asp Thr Arg Thr Met Thr Arg Val Leu Tyr Glu Arg Ala Gln
225 230 235 240

Ala Glu Phe Gly Ser Val Ser Asp Asp Gln Leu Val Tyr Met Ile Thr
245 250 255

Gly Leu Ile Phe Ala Gly His Asp Thr Thr Gly Ser Phe Leu Gly Phe
260 265 270

Leu Leu Ala Glu Val Leu Ala Gly Arg Leu Ala Ala Asp Ala Asp Gly
275 280 285

Asp Ala Ile Ser Arg Phe Val Glu Glu Ala Leu Arg His His Pro Pro
290 295 300

Val Pro Tyr Ser Leu Trp Arg Phe Ala Ala Thr Glu Val Val Ile Arg
305 310 315 320

Gly Val Arg Leu Pro Arg Gly Ala Pro Val Leu Val Asp Ile Glu Gly
325 330 335

Thr Asn Thr Asp Gly Arg His His Asp Ala Pro His Ala Phe His Pro
340 345 350

Asp Arg Pro Ser Arg Arg Arg Leu Thr Phe Gly Asp Gly Pro His Tyr
355 360 365

Cys Ile Gly Glu Gln Leu Ala Gln Leu Glu Ser Arg Thr Met Ile Gly
370 375 380

Val Leu Arg Ser Arg Phe Pro Gln Ala Arg Leu Ala Val Pro Tyr Glu
385 390 395 400

Glu Leu Arg Trp Cys Arg Lys Gly Ala Gln Thr Ala Arg Leu Thr Asp
405 410 415

Leu Pro Val Trp Leu Arg
420

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3013 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCAGGCGGTA CCGCCGACCC GCTGCATCCC CCGCACCGCC GTCCCCCCCC AGGGCATCTC 60

CCGTCGGGTT ACGGGAAGGG GGCCGGGGTA CCCGGTCGTC ACGGGAGGGC TGGGACGAGT 120

GCCCCCGACC CACTGCGTTC CAGCCACTCC CGGTACGCCG GGGCCTGCCG GGCGACCTCC 180

CCGTAGGCCT CCGCGAGGTC GGGGTAGACG CCCTCCAGTT CCGTGTCCGT GCGGGCCGCC 240

AGCAGCAGCC GTACGCCGAG CGGGTCGCCG TGCAGCCGGC GGACGGCCGT CTCGGCGCGG 300

GAGGGCGAGG TCGGCTGGAC CACGGTGACG ACCTCGCCGG TGGCGACCAG GTACGCGGCG 360

GAGTGGTAGT CCCCGTGCAG GATGCGCGAG TCGAGTCCCT CGGCGCGCAG GACCCGGCGC 420

ACCGCGTTCC ACTCGCCGTC GACGGTGGGG TCGATCATCC AGCGGGTCGT GGGCCAGGTC 480

GGCGAGGCGT ACGACGTGGC TTCGGCGGCC GGGTGGTCGG CCGGCAGGCA GACGAACTGC 540

GGTTCCCGCT GGACCAGTAC GCGGACCCGG AGCCCTTCGG GGACGCGCAG GCTGCCCTCG 600

ACCTCGTGCA CGAAGGCGAC GTCGAGGTGG CCGTCGGCCA CCATGCGCAG CAGGGCGTTG 660

GCGGAGACGT CCATGTGCAG GGTGGGTTCC TGCCAGTGCC GGAGCCGGCG CAGCCAGCCC 720

GCCAGGGCCC GGCTGGCCGT GGAGCCGACG CGCAGGCTGG CGTCCGCGAC GGCGGCGGCG 780

CGGGCCTCGC TGACGAGGGA GCACAATTCG GCCACCAGGG GGCGGGCACG ACTGAGAACC 840

AGCCGGCCCA GCGGTGTGGG GCGGCAGCCG GTGCGGGCCC GGACGAACAG GGCACGGCCC 900

AGCTCGTGTT GGATGCGCCG CAGCTGCGTG CTCAACGAGG GCTGTGTCAC TCCCAGTTGG 960

CGTGCCGCGC GGTGCAGGCT GCCGGTGTCG GCGATGGCGC ACAGCGCCCT GAGGTGCCTG 1020

ACCTCAAGCT CCATGTCCTG GGAGGGTAAG GCGGAAGTTC AGCTTTCACC AGACATACAA 1080

AATGGCGACC GATCAGGACC ATCGGGCCTT CACGGCGCGA GGCGTCGGCC CGGATCGGCA 1140

GGGGCCCCGG CCGGGGCCGC CGGGCAGGGC GGCGCAGGTG GGGACGGAGG GGGATAGGGC 1200

GGCCCTATCG GCGGTTGCCA TCATCACAAC GGCCGTACGG GCACGGACAC TCACGATGTC 1260

TGACTCATCC CCCCACCTCG AGGAGTCATC GATGCGCATG CGGAGGGGTG CCTCATCAGC 1320

GGCCCTATCG GCGGTTGCCA TCATCACAAC GGCCGTACGG GCACGGACAC TCACGATGTC 1380

TGACTCATCC CCCCACCTCG AGGAGTCATC GATGCGCATG CGGAGGGGTG CCTCATGAGC 1440

GCGGGCGGAC CCGCCTGGGT CATCACCGAT GACGCCCTCG CCCGCGAGGT GCTGGCCGAT 1500

CCCCGGTTCG TGAAGGACCC CGATCTCGCG CCCACCGCCT GGCGGGGGGT GGACGACGGT 1560

CTCGACATCC CCGTTCCGGA GCTGCGTCCG TTCACGCTCA TCGCCGTGGA CGGTGAGGAC 1620

CACCGCCGTC TGCGCCGCAT CCACGCACCG GCGTTCAACC CGCGCCGGCT GGCCGAGCGG 1680

ACGGATCGCA TCGCCGCCAT CGCCGACCGG CTGCTCACCG AACTCGCCGA CTCCTCCGAC 1740

CGGTCGGGCG AACCGGCCGA GCTGATCGGC GGCTTCGCGT ACCACTTCCC GCTGTTGGTC 1800

ATCTGCGAAC TGCTCGGCGT GCCGGTCACC GATCCGGCAA TGGCCCGCGA GGCCGTCGGC 1860

GTGCTCAAGG CACTCGGCCT CGGCGGCCCG CAGAGCGCCG GCGGTGACGG CACGGACCCT 1920

GCCGGGGACG TGCCGGACAC GTCGGCGCTG GAGAGCCTTC TCCTCGAAGC CGTGCACGCG 1980

GCCCGGCGGA AAGACACCCG GACCATGACC CGCGTGCTCT ATGAACGCGC ACAGGCAGAG 2040

TTCGGCTCGG TCTCCGACGA CCAGCTCGTC TACATGATCA CCGGACTCAT CTTCGCCGGC 2100

CACGACACCA CCGGCTCGTT CCTGGGCTTC CTGCTTGCGG AGGTCCTGGC GGGCCGTCTC 2160

GCGGCGGACG CCGACGGGGA CGCCATCTCC CGGTTCGTGG AGGAGGCGCT GCGCCACCAC 2220

CCGCCGGTGC CCTACACGTT GTGGAGGTTC GCTGCCACGG AGGTGGTCAT CCGCGGTGTC 2280

CGGCTGCCCC GCGGAGCGCC GGTACTGGTG GACATCGAGG GCACCAACAC CGACGGCCGC 2340

CATCACGACG CCCCGCACGC TTTCCACCCG GACCGCCCTT CGAGGCGGCG GCTCACCTTC 2400

GGCGACGGGC CGCACTACTG CATCGGGGAG CAGCTCGCCC AGCTGGAATC GCGCACGATG 2460

ATCGGCGTAC TGCGCAGCAG GTTCCCCCAA GCCCGACTGG CCGTGCCGTA CGAGGAGTTG 2520

CGGTGGTGCA GGAAGGGGGC CCAGACAGCG CGGCTCACTG ACCTGCCCGT CTGGCTGCGT 2580

TGATGGGCCG ACCGCGACCC GGCACGGGAC CGCCCACCGC CCATCGCGCG GTGGGCGGTC 2640

CCGTGCCGGT CGCCCGGTGC GGTCCTCTCC CGACGCTCGC TCCCCCTGTG ACTTTCTCAC 2700

ATCGAGACGT GACGAAATAA TCCCAGCAAG TGCCATGCAC ACTTTCATGG CGGACATTCA 2760

CTTGCGAGGA TGGAGTGAGC ACACGGGGCC GCCCGAGACA CCCTACGGCC GCCGGAAGTA 2820

TGCCACCTGT TGACGCGAAT GGAACGCCAC AGAGGGAGCA CCGCCAATGC AGATCAATAT 2880

GTTGGGCCCG CTCGTTGCAC ATCACAATGG CACGTCGGTG ACCCCGATAG CCAGAAAACC 2940

CCGGCAGGTA TTCTCACTGC TCGCTCTTCA GGCAGGAACC GTCGTTCCGG TCCCCGCGCT 3000

GATGGAGGAG CTC 3013

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2081 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 227..1649

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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GACAATCTGT GTGGGCACTC GACCGGAATT GGGCATCGAT TAACTTTATT ATTAAAAATT 180

AAAGAGGTAT ATATTAATGT ATCGATTAAA TAAGGAGGAA TAAACC ATG GGG GGT 235
Met Gly Gly
1

TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT GGT GGA CAG 283
Ser His His His His His His Gly Met Ala Ser Met Thr Gly Gly Gln
5 10 15

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CAA ATG GGT CGG GAT CTG TAC GAC GAT GAC GAT AAG GAT CGA TGG ATC          331
Gln Met Gly Arg Asp Leu Tyr Asp Asp Asp Asp Lys Asp Arg Trp Ile
 20                  25                  30                  35

CGA CCT CGA GAT CTG CAG ATG GTA CCA TAT GGG AAT TCG GAG GGG TGC          379
Arg Pro Arg Asp Leu Gln Met Val Pro Tyr Gly Asn Ser Glu Gly Cys
                 40                  45                  50

CTC ATG AGC GGC GAG GCG CCG CGG GTG GCC GTC GAC CCG TTC TCG TGT          427
Leu Met Ser Gly Glu Ala Pro Arg Val Ala Val Asp Pro Phe Ser Cys
             55                  60                  65

CCC ATG ATG ACC ATG CAG CGC AAA CCC GAG GTG CAC GAC GCA TTC CGA          475
Pro Met Met Thr Met Gln Arg Lys Pro Glu Val His Asp Ala Phe Arg
         70                  75                  80

GAG GCG GGC CCC GTC GTC GAG GTG AAC GCC CCC GCG GGC GGA CCC GCC          523
Glu Ala Gly Pro Val Val Glu Val Asn Ala Pro Ala Gly Gly Pro Ala
     85                  90                  95

TGG GTC ATC ACC GAT GAC GCC CTC GCC CGC GAG GTG CTG GCC GAT CCC          571
Trp Val Ile Thr Asp Asp Ala Leu Ala Arg Glu Val Leu Ala Asp Pro
100                 105                 110                 115

CGG TTC GTG AAG GGA CCC GAT CTC GCG CCC ACC GCC TGG CGG GGG GTG          619
Arg Phe Val Lys Gly Pro Asp Leu Ala Pro Thr Ala Trp Arg Gly Val
                120                 125                 130

GAC GAC GGT CTC GAC ATC CCC GTT CCG GAG CTG CGT CCG TTC ACG CTC          667
Asp Asp Gly Leu Asp Ile Pro Val Pro Glu Leu Arg Pro Phe Thr Leu
            135                 140                 145

ATC GCC GTG GAC GGT GAG GAC CAC CGG CGT CTG CGC CGC ATC CAC GCA          715
Ile Ala Val Asp Gly Glu Asp His Arg Arg Leu Arg Arg Ile His Ala
        150                 155                 160

CCG GCG TTC AAC CCG CGC CGG CTG GCC GAG CGG ACG GAT CGC ATC GCC          763
Pro Ala Phe Asn Pro Arg Arg Leu Ala Glu Arg Thr Asp Arg Ile Ala
    165                 170                 175

GCC ATC GCC GAC CGG CTG CTC ACC GAA CTC GCC GAC TCC TCC GAC CGG          811
Ala Ile Ala Asp Arg Leu Leu Thr Glu Leu Ala Asp Ser Ser Asp Arg
180                 185                 190                 195

TCG GGC GAA CCG GCC GAG CTG ATC GGC GGC TTC GCG TAC CAC TTC CCG          859
Ser Gly Glu Pro Ala Glu Leu Ile Gly Gly Phe Ala Tyr His Phe Pro
                200                 205                 210

CTG TTG GTC ATC TGC GAA CTG CTC GGC GTG CCG GTC ACC GAT CCG GCA          907
Leu Leu Val Ile Cys Glu Leu Leu Gly Val Pro Val Thr Asp Pro Ala
            215                 220                 225

ATG GCC CGC GAG GCC GTC GGC GTG CTC AAG GCA CTC GGC CTC GGC GGC          955
Met Ala Arg Glu Ala Val Gly Val Leu Lys Ala Leu Gly Leu Gly Gly
        230                 235                 240

CCG CAG AGC GCC GGC GGT GAC GGC ACG GAC CCT GCC GGG GAC GTG CCG         1003
Pro Gln Ser Ala Gly Gly Asp Gly Thr Asp Pro Ala Gly Asp Val Pro
    245                 250                 255

GAC ACG TCG GCG CTG GAG AGC CTT CTC CTC GAA GCC GTG CAC GCG GCC         1051
Asp Thr Ser Ala Leu Glu Ser Leu Leu Leu Glu Ala Val His Ala Ala
260                 265                 270                 275

CGG CGG AAA GAC ACC CGG ACC ATG ACC CGC GTG CTC TAT GAA CGC GCA         1099
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```

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                  280                 285                 290

CAG GCA GAG TTC GGC TCG GTC TCC GAC GAC CAG CTC GTC TAC ATG ATC      1147
Gln Ala Glu Phe Gly Ser Val Ser Asp Asp Gln Leu Val Tyr Met Ile
            295                 300                 305

ACC GGA CTC ATC TTC GCC GGC CAC GAC ACC ACC GGC TCG TTC CTG GGC      1195
Thr Gly Leu Ile Phe Ala Gly His Asp Thr Thr Gly Ser Phe Leu Gly
        310                 315                 320

TTC CTG CTT GCG GAG GTC CTG GCG GGC CGT CTC GCG GCG GAC GCC GAC      1243
Phe Leu Leu Ala Glu Val Leu Ala Gly Arg Leu Ala Ala Asp Ala Asp
    325                 330                 335

GGG GAC GCC ATC TCC CGG TTC GTG GAG GAG GCG CTG CGC CAC CAC CCG      1291
Gly Asp Ala Ile Ser Arg Phe Val Glu Glu Ala Leu Arg His His Pro
340                 345                 350                 355

CCG GTG CCC TAC TCG TTG TGG AGG TTC GCT GCC ACG GAG GTG GTC ATC      1339
Pro Val Pro Tyr Ser Leu Trp Arg Phe Ala Ala Thr Glu Val Val Ile
                360                 365                 370

CGC GGT GTC CGG CTG CCC CGC GGA GCG CCG GTA CTG GTG GAC ATC GAG      1387
Arg Gly Val Arg Leu Pro Arg Gly Ala Pro Val Leu Val Asp Ile Glu
            375                 380                 385

GGC ACC AAC ACC GAC GGC CGC CAT CAC GAC GCC CCG CAC GCT TTC CAC      1435
Gly Thr Asn Thr Asp Gly Arg His His Asp Ala Pro His Ala Phe His
        390                 395                 400

CCG GAC CGC CCT TCG AGG CGG CGG CTC ACC TTC GGC GAC GGG CCG CAC      1483
Pro Asp Arg Pro Ser Arg Arg Arg Leu Thr Phe Gly Asp Gly Pro His
    405                 410                 415

TAC TGC ATC GGG GAG CAG CTC GCC CAG CTG GAA TCG CGC ACG ATG ATC      1531
Tyr Cys Ile Gly Glu Gln Leu Ala Gln Leu Glu Ser Arg Thr Met Ile
420                 425                 430                 435

GGC GTA CTG CGC AGC AGG TTC CCC CAA GCC CGA CTG GCC GTG CCG TAC      1579
Gly Val Leu Arg Ser Arg Phe Pro Gln Ala Arg Leu Ala Val Pro Tyr
                440                 445                 450

GAG GAG TTG CGG TGG TGC AGG AAG GGG GCC CAG ACA GCG CGG CTC ACT      1627
Glu Glu Leu Arg Trp Cys Arg Lys Gly Ala Gln Thr Ala Arg Leu Thr
            455                 460                 465

GAC CTG CCC GTC TGG CTG CGT T GATGGGCCGA CCGCGACCCG GCACGGGACC       1679
Asp Leu Pro Val Trp Leu Arg
        470

GCCCACCGCC CATCGCGCGG TGGGCGGTCC CGTGCCGGTC GCCCGGTGCG GTCCTCTCCC    1739

GACGCTCGCT CCCCCTGTGA CTTTCTCACA TCGAGACGTG ACGAAATAAT CCCAGCAAGT    1799

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CCCGAGACAC CCTACGGCCG CCGGAAGTAT GCCACCTGTT GACGCGAATG GAACGCCACA    1919

GAGGGAGCAC CGGCAATGCA GATCAATATG TTGGGCCCGC TCGTTGCACA TCACAATGGC    1979

ACGTCGGTGA CCCCGATAGC CAGAAAACCC CGGCAGGTAT TCTCACTGCT CGCTCTTCAG    2039

GCAGGAACCG TCGTTCCGGT CCCCGCGCTG ATGGAGGAGC TC                       2081
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(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 474 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Gly Gly Ser His His His His His His Gly Met Ala Ser Met Thr
1 5 10 15

Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp Asp Asp Lys Asp
20 25 30

Arg Trp Ile Arg Pro Arg Asp Leu Gln Met Val Pro Tyr Gly Asn Ser
35 40 45

Glu Gly Cys Leu Met Ser Gly Glu Ala Pro Arg Val Ala Val Asp Pro
50 55 60

Phe Ser Cys Pro Met Met Thr Met Gln Arg Lys Pro Glu Val His Asp
65 70 75 80

Ala Phe Arg Glu Ala Gly Pro Val Val Glu Val Asn Ala Pro Ala Gly
85 90 95

Gly Pro Ala Trp Val Ile Thr Asp Asp Ala Leu Ala Arg Glu Val Leu
100 105 110

Ala Asp Pro Arg Phe Val Lys Gly Pro Asp Leu Ala Pro Thr Ala Trp
115 120 125

Arg Gly Val Asp Asp Gly Leu Asp Ile Pro Val Pro Glu Leu Arg Pro
130 135 140

Phe Thr Leu Ile Ala Val Asp Gly Glu Asp His Arg Arg Leu Arg Arg
145 150 155 160

Ile His Ala Pro Ala Phe Asn Pro Arg Arg Leu Ala Glu Arg Thr Asp
165 170 175

Arg Ile Ala Ala Ile Ala Asp Arg Leu Leu Thr Glu Leu Ala Asp Ser
180 185 190

Ser Asp Arg Ser Gly Glu Pro Ala Glu Leu Ile Gly Gly Phe Ala Tyr
195 200 205

His Phe Pro Leu Leu Val Ile Cys Glu Leu Leu Gly Val Pro Val Thr
210 215 220

Asp Pro Ala Met Ala Arg Glu Ala Val Gly Val Leu Lys Ala Leu Gly
225 230 235 240

Leu Gly Gly Pro Gln Ser Ala Gly Gly Asp Gly Thr Asp Pro Ala Gly
245 250 255

Asp Val Pro Asp Thr Ser Ala Leu Glu Ser Leu Leu Leu Glu Ala Val
260 265 270

His Ala Ala Arg Arg Lys Asp Thr Arg Thr Met Thr Arg Val Leu Tyr
275 280 285

Glu Arg Ala Gln Ala Glu Phe Gly Ser Val Ser Asp Asp Gln Leu Val
290 295 300

Tyr Met Ile Thr Gly Leu Ile Phe Ala Gly His Asp Thr Thr Gly Ser
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Phe Leu Gly Phe Leu Leu Ala Glu Val Leu Ala Gly Arg Leu Ala Ala
325 330 335

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340 345 350

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355 360 365

Val Val Ile Arg Gly Val Arg Leu Pro Arg Gly Ala Pro Val Leu Val
370 375 380

Asp Ile Glu Gly Thr Asn Thr Asp Gly Arg His His Asp Ala Pro His
385 390 395 400

Ala Phe His Pro Asp Arg Pro Ser Arg Arg Arg Leu Thr Phe Gly Asp
405 410 415

Gly Pro His Tyr Cys Ile Gly Glu Gln Leu Ala Gln Leu Glu Ser Arg
420 425 430

Thr Met Ile Gly Val Leu Arg Ser Arg Phe Pro Gln Ala Arg Leu Ala
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Val Pro Tyr Glu Glu Leu Arg Trp Cys Arg Lys Gly Ala Gln Thr Ala
450 455 460

Arg Leu Thr Asp Leu Pro Val Trp Leu Arg
465 470

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 443 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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20 25 30

Pro Phe Ser Cys Pro Met Met Thr Met Gln Arg Lys Pro Glu Val His
35 40 45

Asp Ala Phe Arg Glu Ala Gly Pro Val Val Glu Val Asn Ala Pro Ala
50 55 60

Gly Gly Pro Ala Trp Val Ile Thr Asp Asp Ala Leu Ala Arg Glu Val
65 70 75 80

Leu Ala Asp Pro Arg Phe Val Lys Asp Pro Asp Leu Ala Pro Thr Ala
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Ser Ser Asp Arg Ser Gly Glu Pro Ala Glu Leu Ile Gly Gly Phe Ala
165 170 175

Tyr His Phe Pro Leu Leu Val Ile Cys Glu Leu Leu Gly Val Pro Val
180 185 190

Thr Asp Pro Ala Met Ala Arg Glu Ala Val Gly Val Leu Lys Ala Leu
195 200 205

Gly Leu Gly Gly Pro Gln Ser Ala Gly Gly Asp Gly Thr Asp Pro Ala
210 215 220

Gly Asp Val Pro Asp Thr Ser Ala Leu Glu Ser Leu Leu Leu Glu Ala
225 230 235 240

Val His Ala Ala Arg Arg Lys Asp Thr Arg Thr Met Thr Arg Val Leu
245 250 255

Tyr Glu Arg Ala Gln Ala Glu Phe Gly Ser Val Ser Asp Asp Gln Leu
260 265 270

Val Tyr Met Ile Thr Gly Leu Ile Phe Ala Gly His Asp Thr Thr Gly
275 280 285

Ser Phe Leu Gly Phe Leu Leu Ala Glu Val Leu Ala Gly Arg Leu Ala
290 295 300

Ala Asp Ala Asp Gly Asp Ala Ile Ser Arg Phe Val Glu Glu Ala Leu
305 310 315 320

Arg His His Pro Pro Val Pro Tyr Thr Leu Trp Arg Phe Ala Ala Thr
325 330 335

Glu Val Val Ile Arg Gly Val Arg Leu Pro Arg Gly Ala Pro Val Leu
340 345 350

Val Asp Ile Glu Gly Thr Asn Thr Asp Gly Arg His His Asp Ala Pro
355 360 365

His Ala Phe His Pro Asp Arg Pro Ser Arg Arg Arg Leu Thr Phe Gly
370 375 380

Asp Gly Pro His Tyr Cys Ile Gly Glu Gln Leu Ala Gln Leu Glu Ser
385 390 395 400

Arg Thr Met Ile Gly Val Leu Arg Ser Arg Phe Pro Gln Ala Arg Leu
405 410 415

Ala Val Pro Tyr Glu Glu Leu Arg Trp Cys Arg Lys Gly Ala Gln Thr
420 425 430

Ala Arg Leu Thr Asp Leu Pro Val Trp Leu Arg
435 440

What is claimed is:

1. A method of making doxorubicin comprising the following steps:
 a. providing a culture of a host microorganism transformed with a plasmid which contains *doxA* gene;
 b. adding daunomycin to said cultures;
 c. incubating said cultures in the presence of daunomycin; and extracting doxorubicin from said cultures.

2. The method of claim 1, wherein the host microorganism is bacterial.

3. The method of claim 1, wherein the host microorganism is *Streptomyces*.

4. The method of claim 1 wherein the *doxA* gene is isolated from Streptomyces.

5. The method of claim 1 wherein the *doxA* gene is driven by promoter selected from the group consisting of: *snpA*, *melCI*, or wild type promoter.

6. The method of claim 5, wherein the promoter is *snpA*.

7. The method of claim 6, wherein the promoter is activated by SnpR.

8. A gene which encodes daunomycin C-14 hydroxylase.

9. The gene of claim 8, wherein the gene encodes a protein having the amino acid sequence of Sequence Id. 5.

10. The gene of claim 8, wherein the gene is the *doxA* gene of *Streptomyces*.

11. The gene of claim 8, wherein the gene has the nucleotide sequence of Sequence ID. 4.

12. A genetically engineered host microorganism for converting daunomycin to doxorubicin comprising:
 a. a plasmid, disposed within said microorganism comprising the following elements:
  a *doxA* gene; and
  a promoter driving the *doxA* gene;
 b. a host microorganism.

13. A plasmid comprising the following elements:
a. a *doxA* gene; and
b. a promoter driving the *doxA* gene.

14. The plasmid of claim 13, further comprising an activator for the promoter.

15. The plasmid of claim 13, further comprising a polylinker.

16. The plasmid of claim 15, wherein the polylinker has the following nucleotide sequence:
GCATGCGAATTCAGATCTAGAGCTCAAGCTTTAAACTAGTTAACGCGT SEQ.ID 3

17. A plasmid comprising the following elements:
a. polylinker; and
b. a *snpA* promoter upstream of the polylinker.

18. A method of making doxorubicin comprising the following steps:
providing a culture of *Streptomyces* sp. strain C5;
incubating said culture in the presence of daunomycin; and
extracting doxorubicin from said culture.

19. A method of making doxorubicin comprising the following steps:
a. providing daunomycin C-14 hydroxylase;
b. incubating said daunomycin C-14 hydroxylase in the presence of daunomycin; and
c. extracting doxorubicin from the mixture of step b.

20. A method of making 13-deoxycarminomycin and 13-deoxydaunomycin comprising the following steps:
a. providing a culture of a host microorganism transformed with a plasmid which contains *dauP* gene and *dauK*;
b. incubating said cultures in the presence of ϵ-Rhodomycin D; and
c. extracting 13-deoxycarminomycin and 13-deoxydaunomycin from said cultures.

21. An isolated daunomycin c-14 hydroxylase which converts of daunomycin to doxorubicin.

22. The plasmid of claim 17, wherein the polylinker has the following nucleotide sequence:
GCATGCGAATTCAGATCTAGAGCTCAAGCTTTAAACTAGTTAACGCGT SEQ.ID 3

23. The protein of claim 22, further comprising a leader sequence encoding six histidine residues,.

24. A method of producing 13-dihydrocarminomycin and carminomycin comprising the following steps:

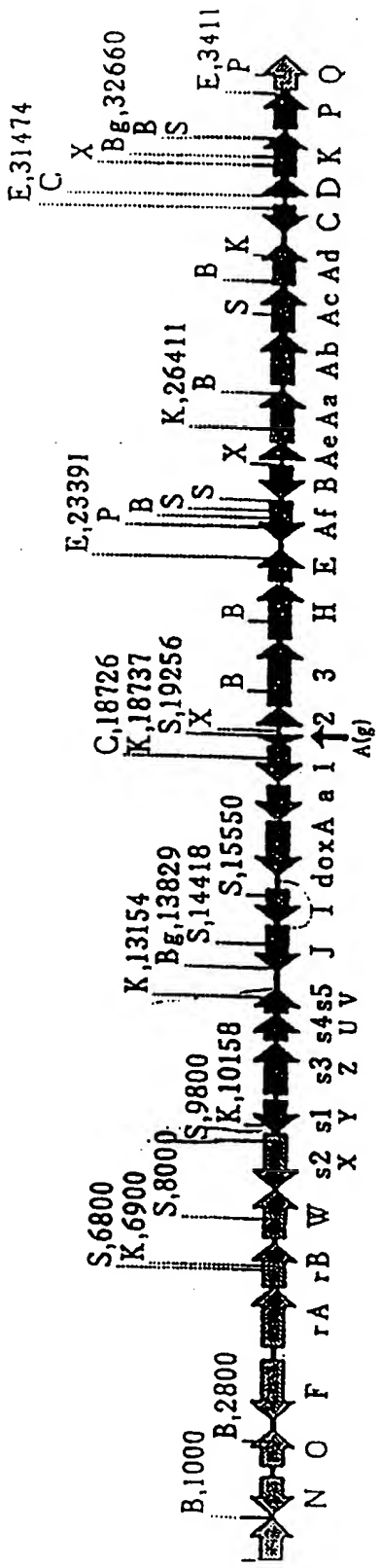
a. incubating daunomycin C-14 hydroxylase with 13-deoxycarminomycin; and

b. extracting 13-dihydrocarminomycin and carminomycin.

25. A method of producing 13-dihydrodaunomycin and daunomycin comprising the following steps:

a. incubating daunomycin C-14 hydroxylase with 13-deoxydaunomycin; and

b. extracting 13-dihydrodaunomycin and daunomycin.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B
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S,19256
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S,15550
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B,1000
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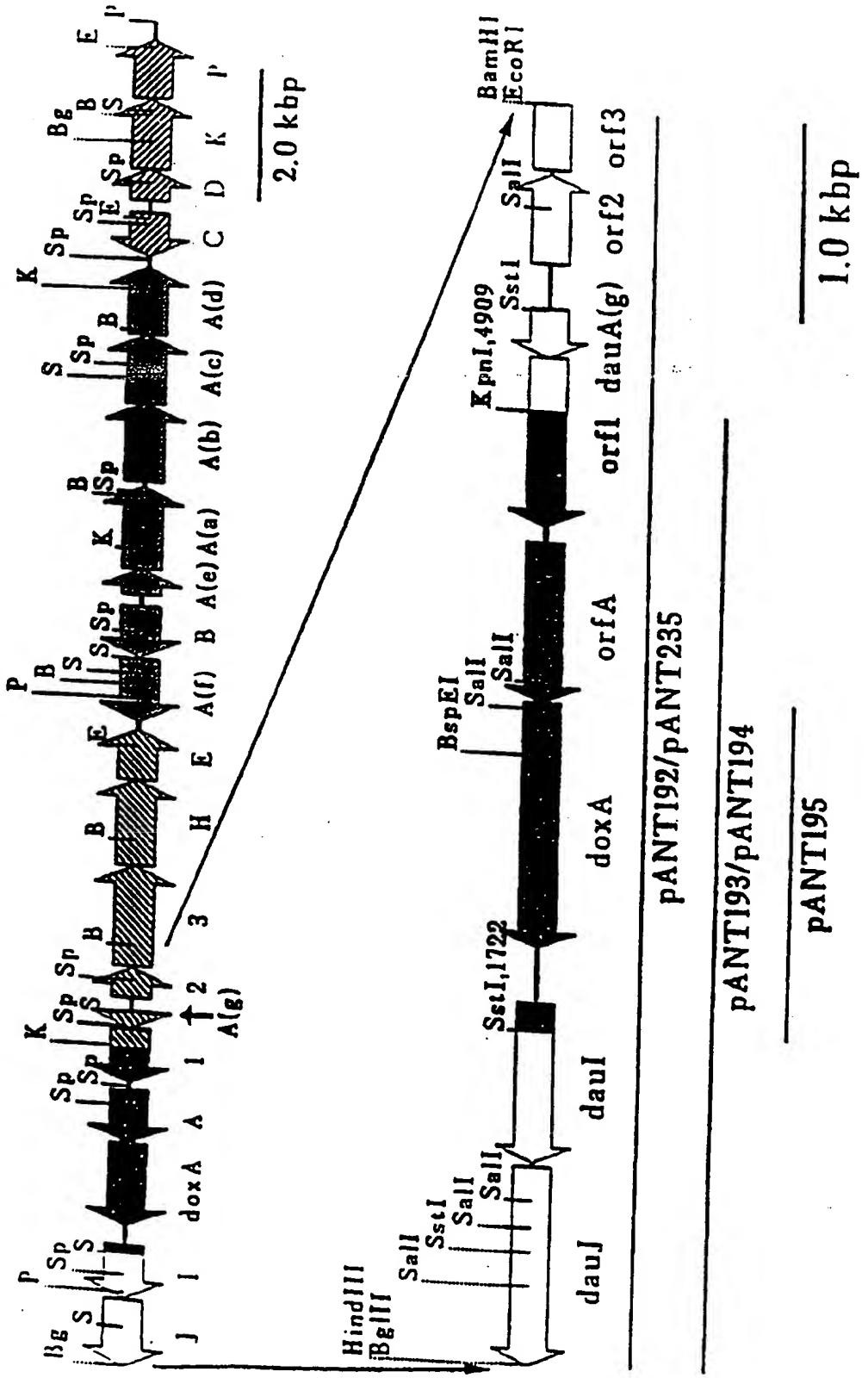
s1
Y
s3
Z
s4s5
U
V

FIGURE 1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1.0 kbp
HindIII
BglII
SalI
SstI
SalI
SalI
dauJ
dauI
SstI,1722
doxA
BspEI
SalI
SalI
orfA
KpnI,4909
SstI
SalI
orf1
dauA(g)
orf2
orf3
BamHI
EcoRI
pANT192/pANT235
pANT193/pANT194
pANT195

FIGURE 2

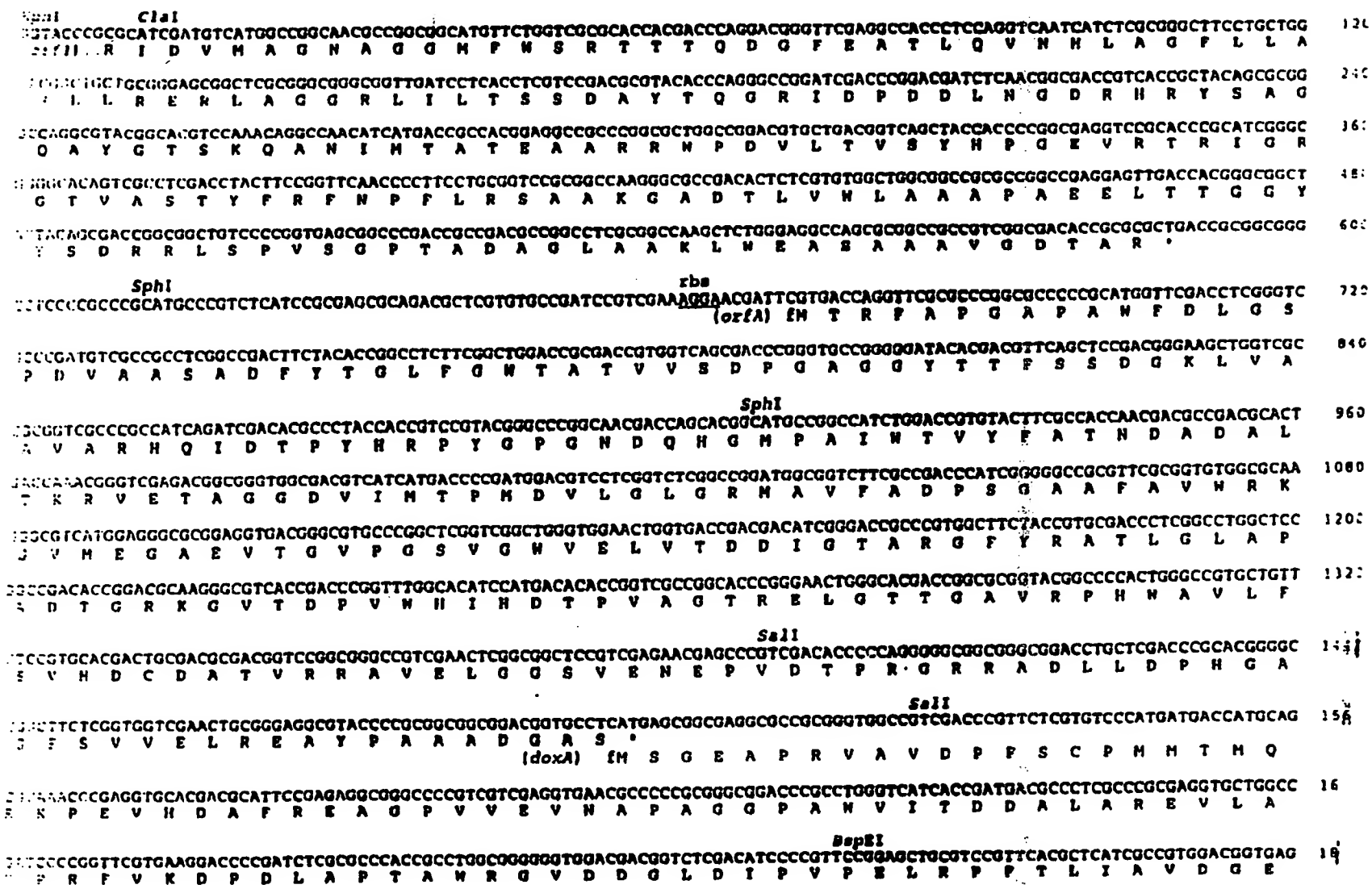

ClaI
SphI
rbs
(orfA) fM T R F A P G A P A W F D L G S
SphI
SalI
SalI
(doxA) fM S G E A P R V A V D P P S C P M M T M Q
BspEI

FIGURE 3

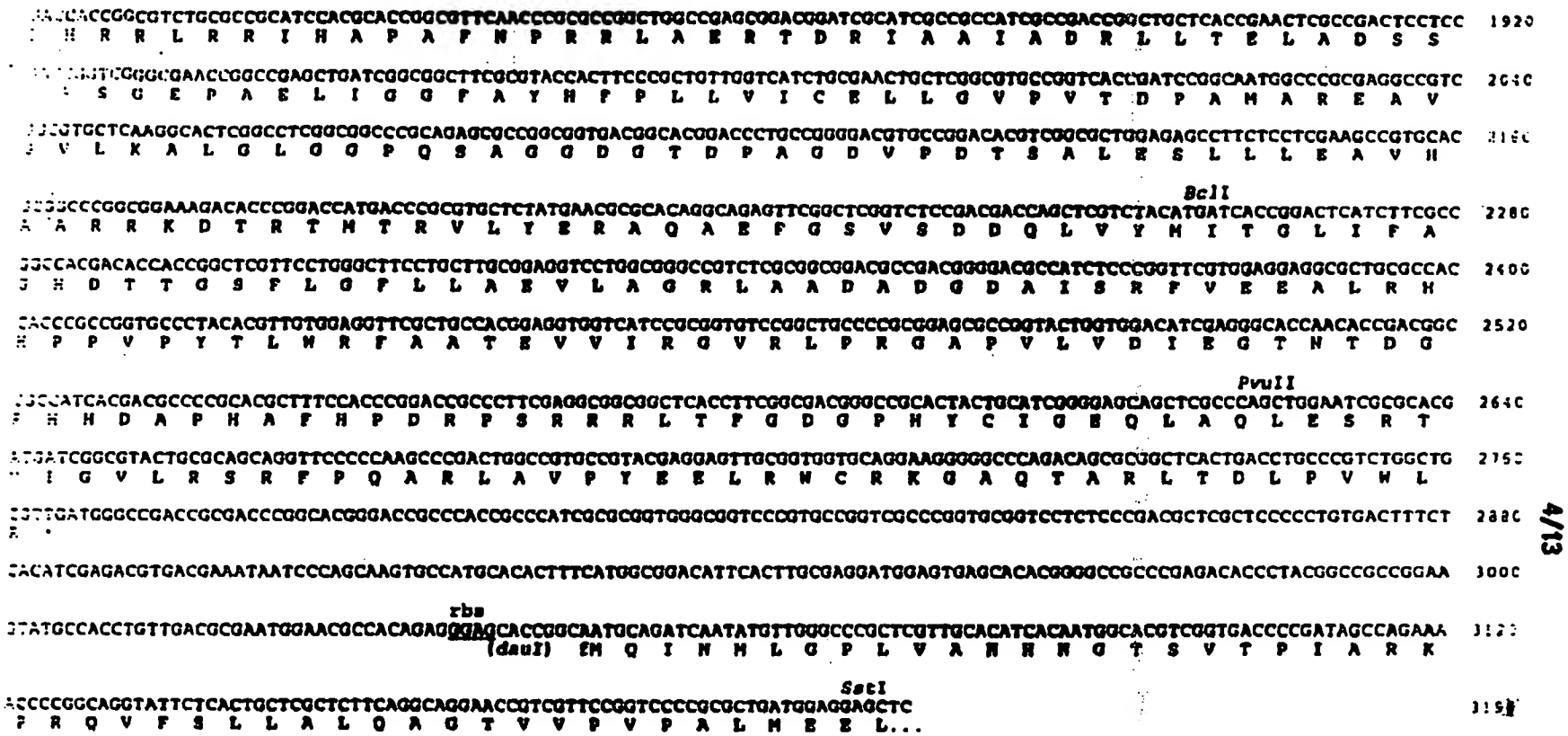
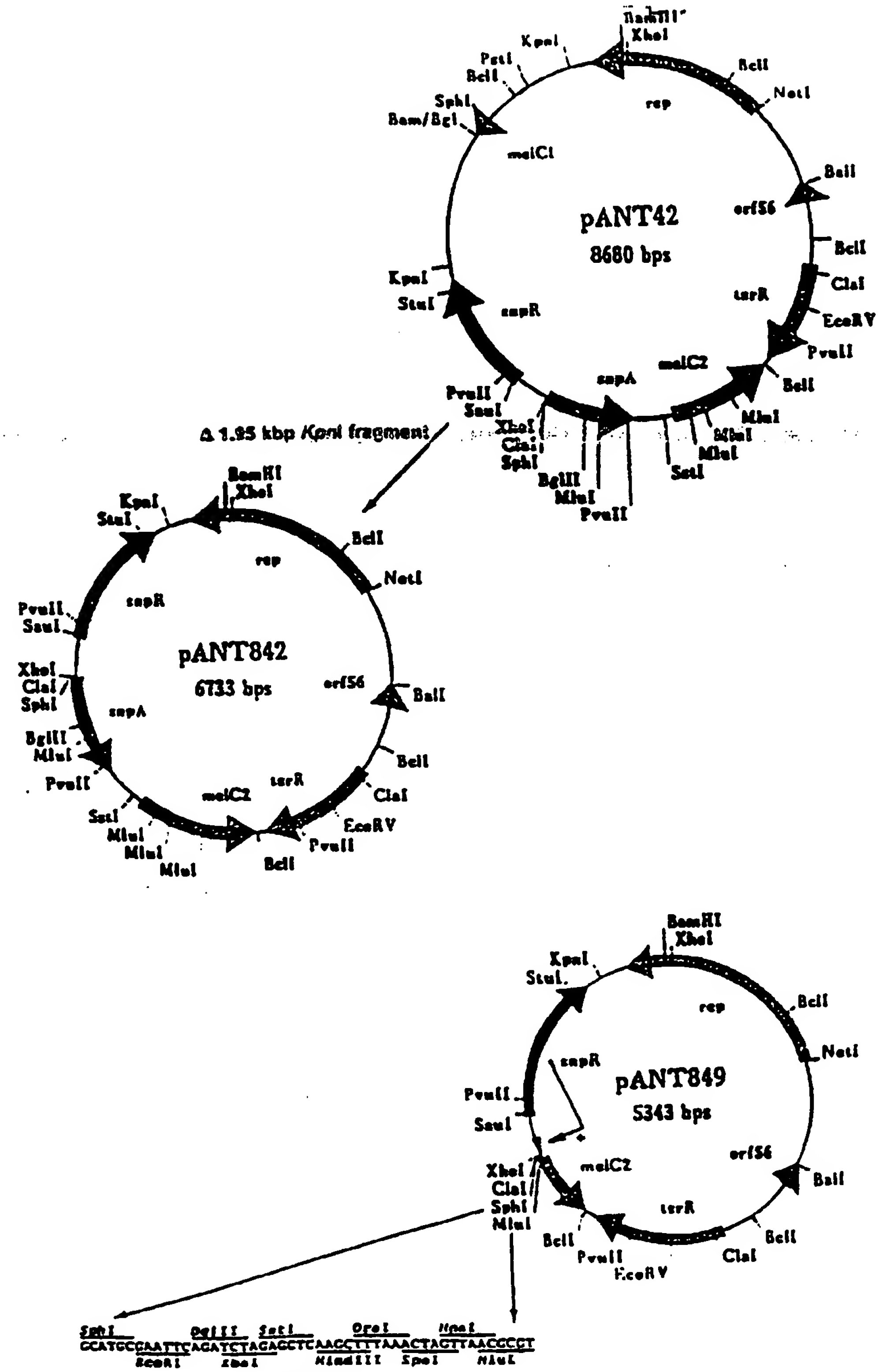
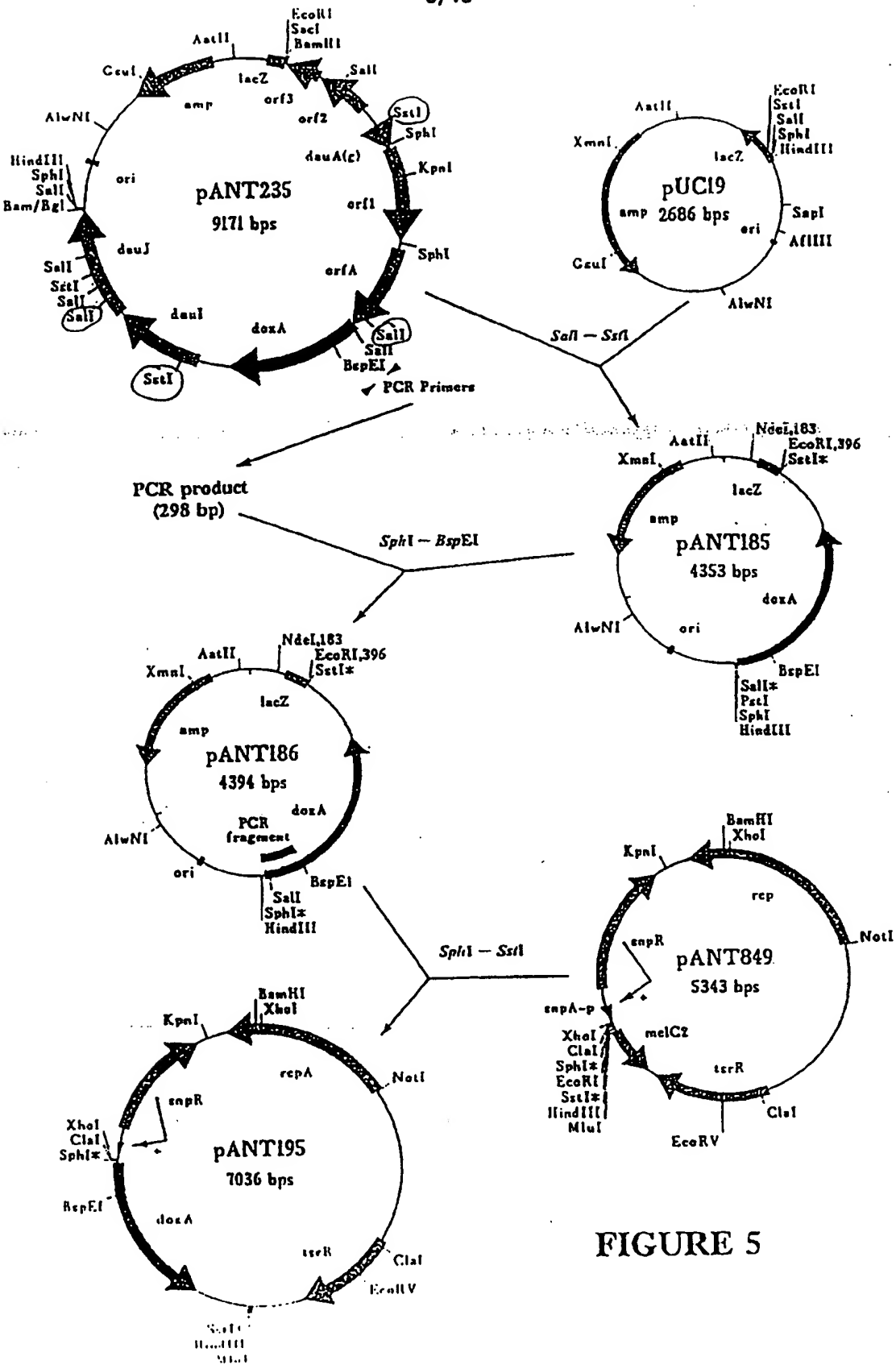


FIGURE 3

FIGURE 4 5/13



P lylinker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pUC19
amp 2686 bps
PCR Primers
PCR product
(298 bp)
SalI — SstI
SphI — BspEI
pANT185
4353 bps
pANT186
4394 bps
PCR fragment
SphI — SstI
pANT849
5343 bps
pANT195
7036 bps

FIGURE 5

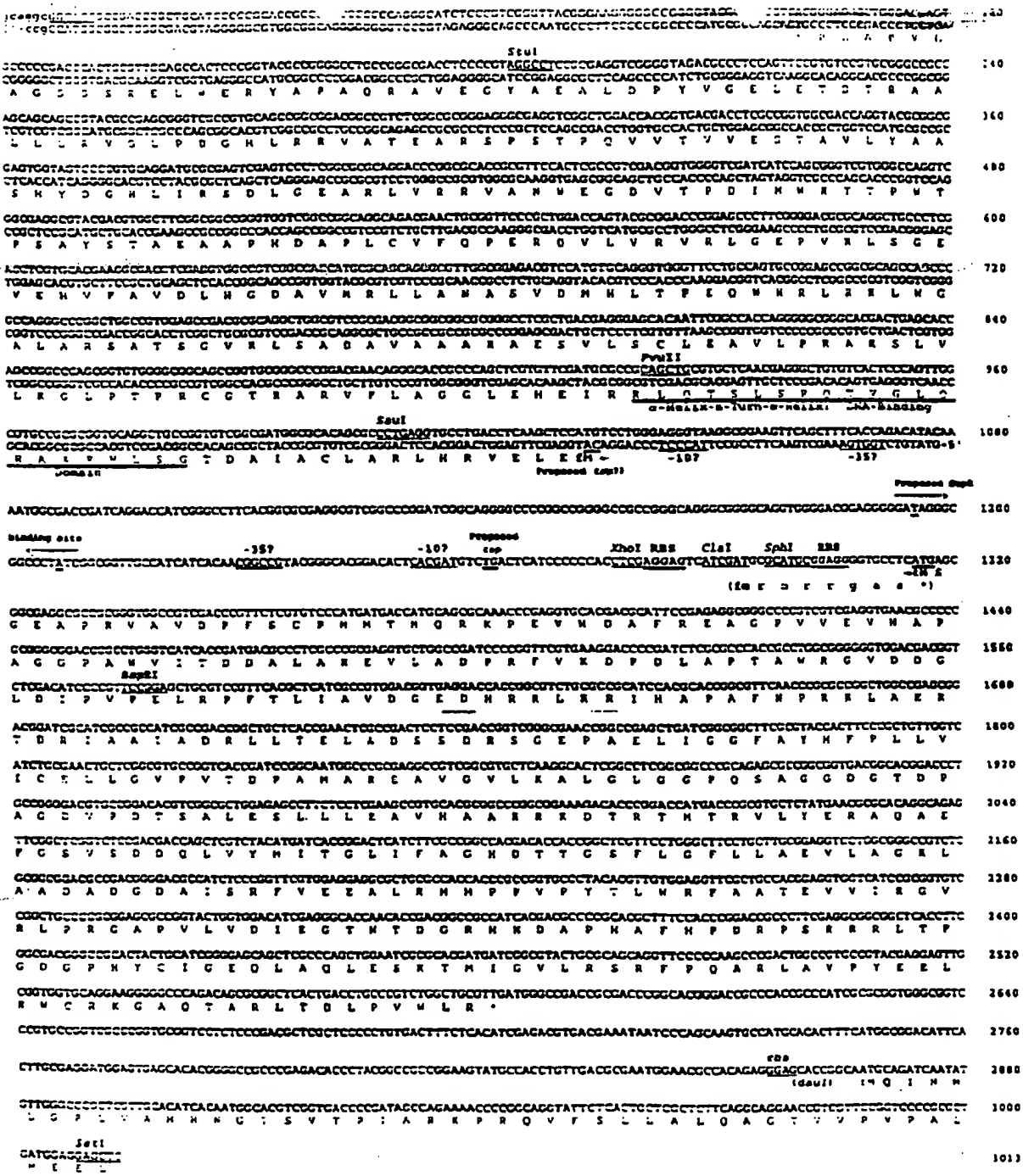
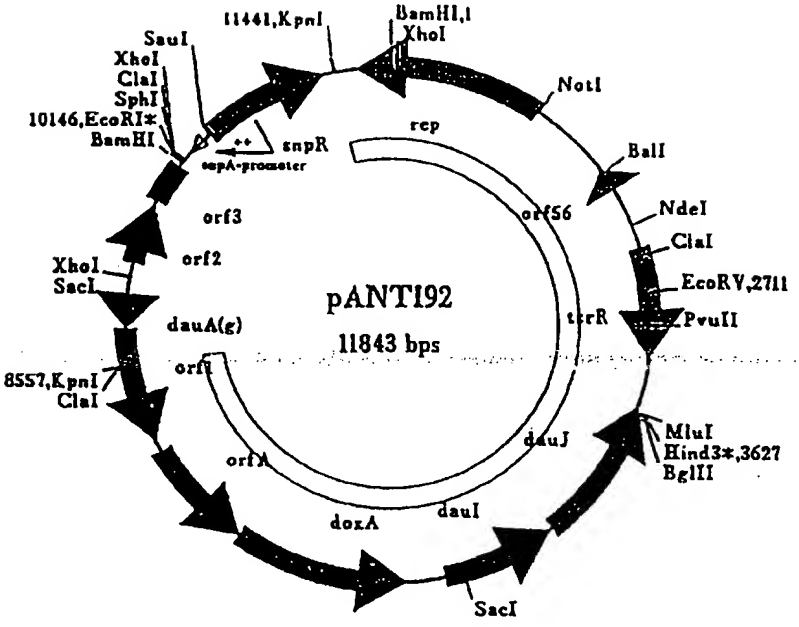


FIGURE 6

FIGURE 7



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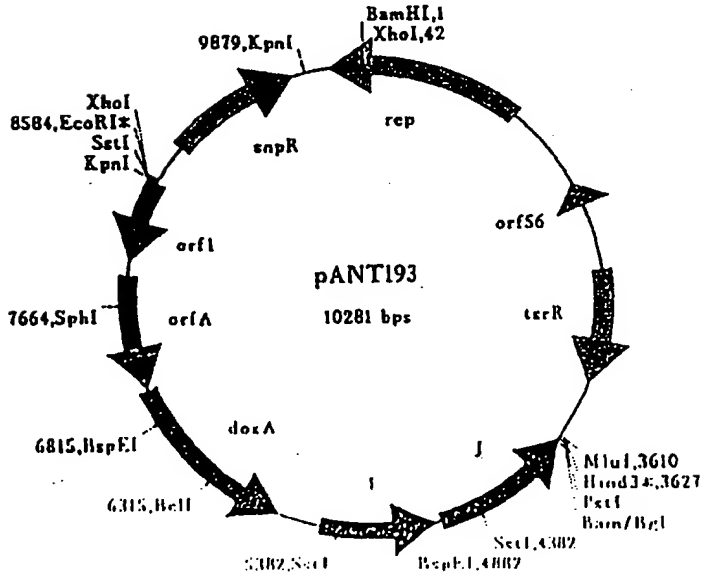
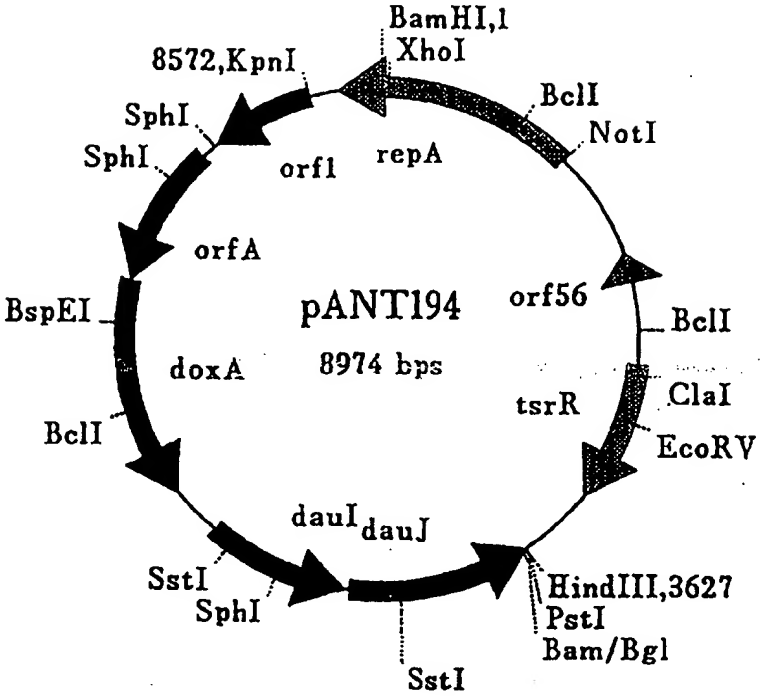
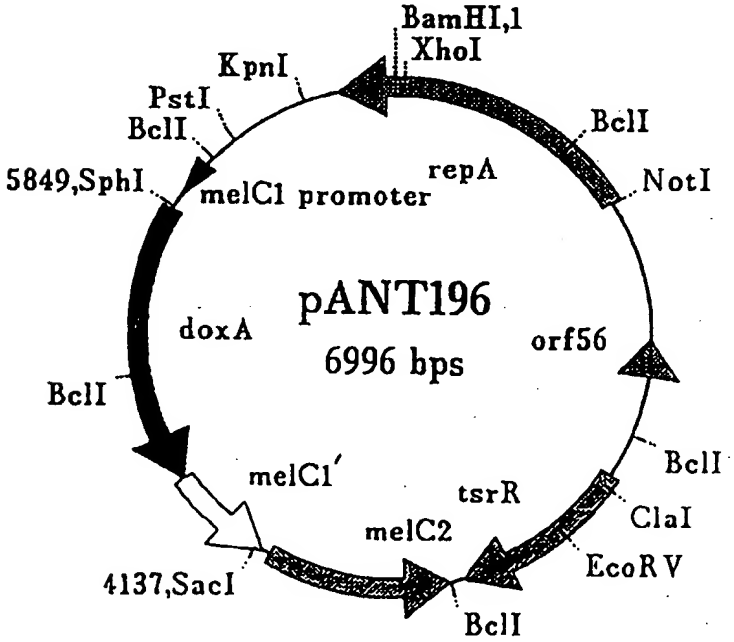


FIGURE 8



pANT196 - pIJ702 + doxA gene expressed from melC1 promoter



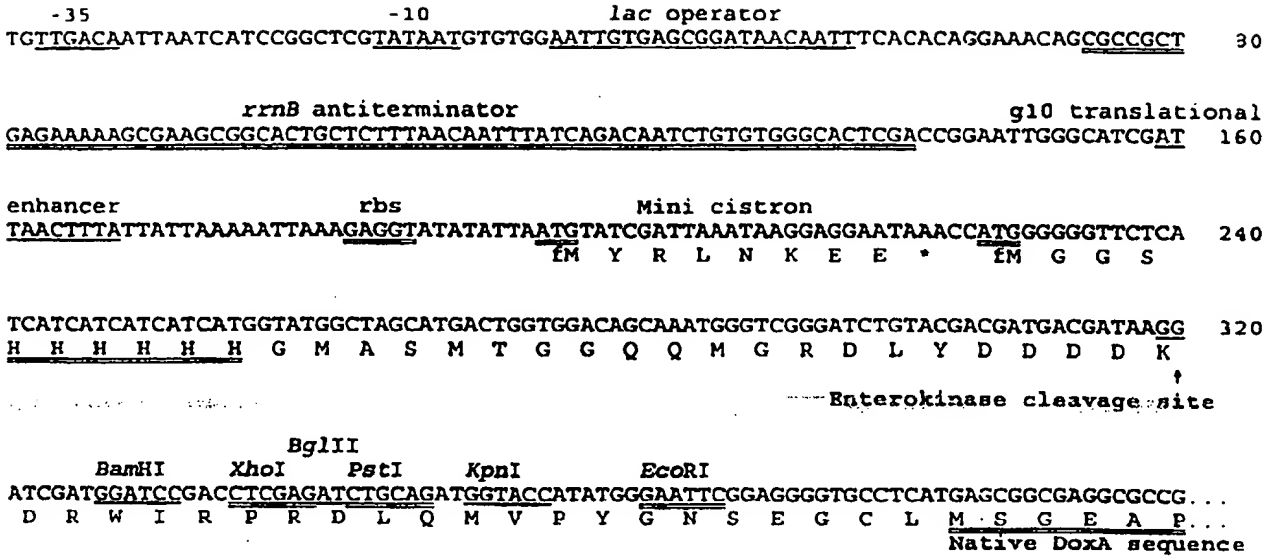
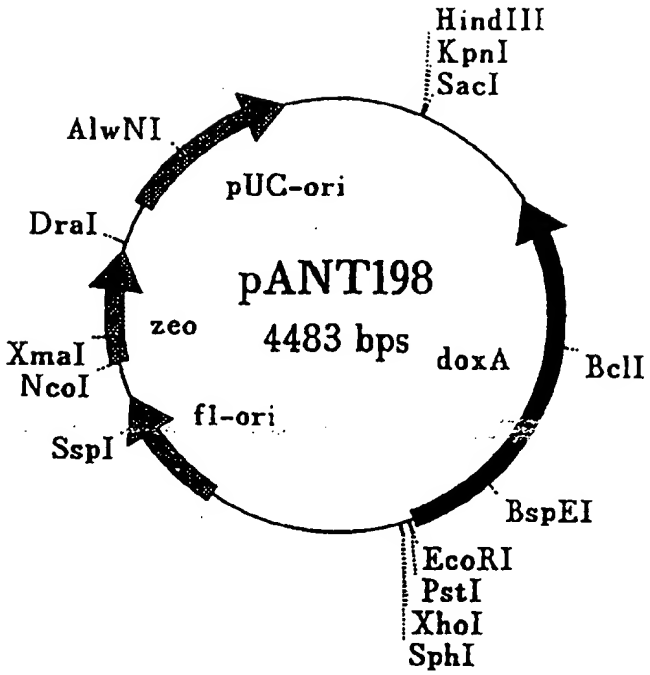

-35
-10
lac operator
TGTTGACAATTAATCATCCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCGCCGCT 30
rrnB antiterminator
g10 translational
GAGAAAAAGCGAAGCGGCACTGCTCTTTAACAATTTATCAGACAATCTGTGTGGGCACTCGACCGGAATTGGGCATCGAT 160
enhancer
rbs
Mini cistron
TAACTTTATTATTAAAAATTAAAGAGGTATATATTAATGTATCGATTAAATAAGGAGGAATAAACCATGGGGGGTTCTCA 240
fM Y R L N K E E * fM G G S
TCATCATCATCATCATGGTATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGGGATCTGTACGACGATGACGATAAGG 320
H H H H H H G M A S M T G G Q Q M G R D L Y D D D D K
Enterokinase cleavage site
BglII
BamHI
XhoI
PstI
KpnI
EcoRI
ATCGATGGATCCGACCTCGAGATCTGCAGATGGTACCATATGGGAATTCGGAGGGGTGCCTCATGAGCGGCGAGGCGCCG...
D R W I R P R D L Q M V P Y G N S E G C L M S G E A P...
Native DoxA sequence

FIGURE 9



pANT199 - doxA in pTrcHis for Expression as a Fusion Protein in Escherichia coli

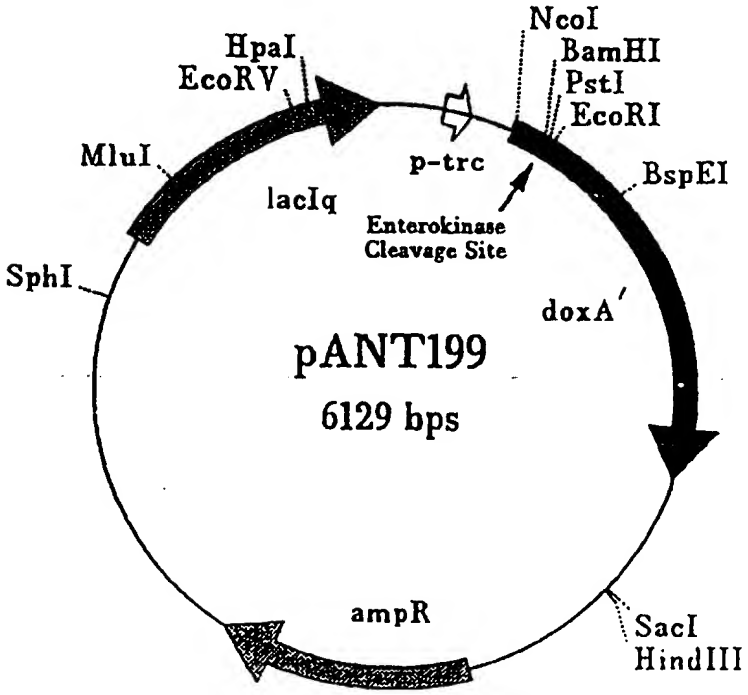


FIGURE 10

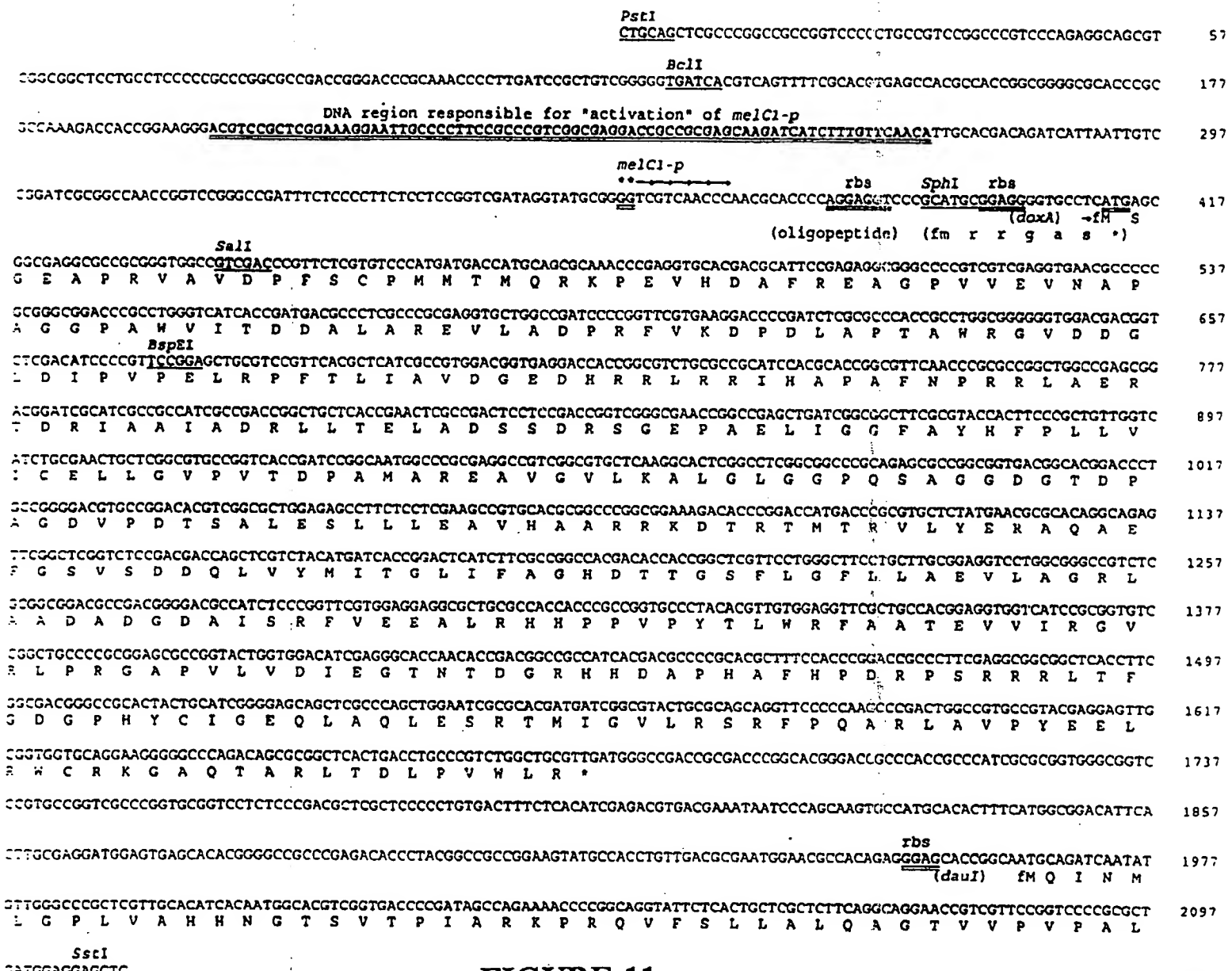
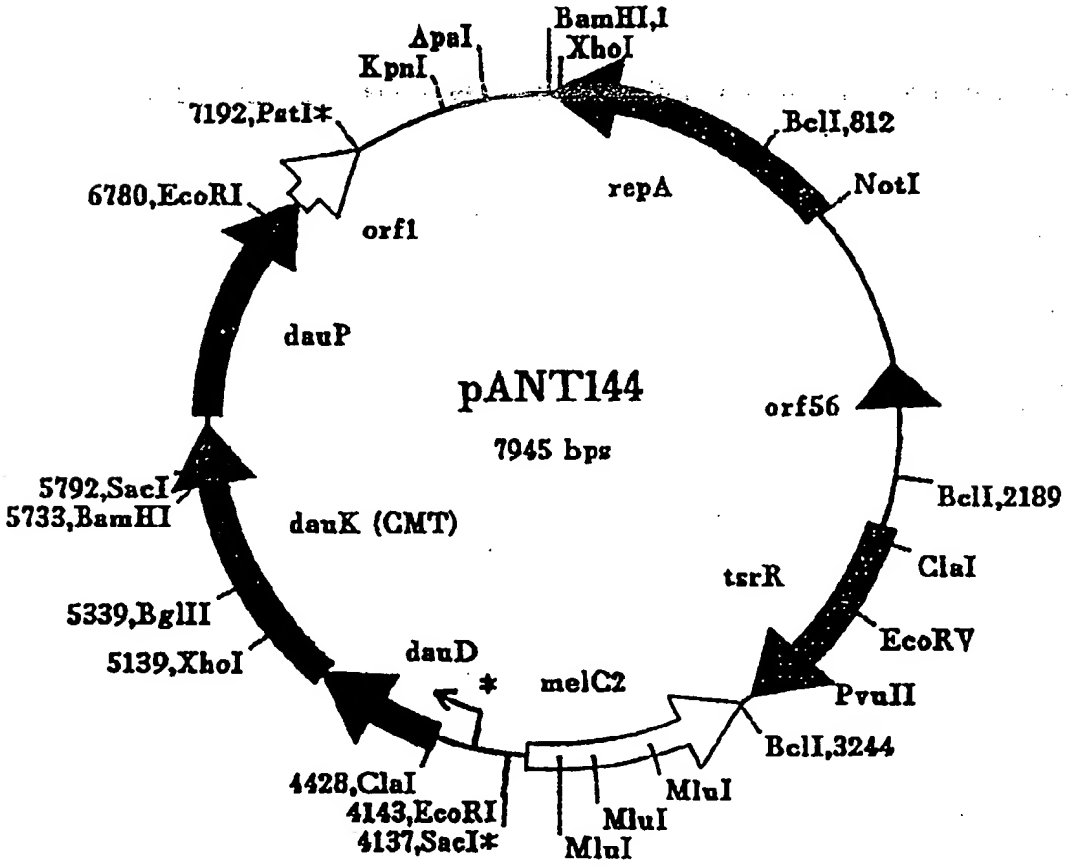

PstI
CTGCAGCTCGCCCGGCCGCCGGTCCCCCTGCCGTCCGGCCCGTCCCAGAGGCAGCGT 57
BclI
CGGCGGCTCCTGCCTCCCCCGCCCGGCGCCGACCGGGACCCGCAAACCCCTTGATCCGCTGTCGGGGGTGATCACGTCAGTTTTCGCACCTGAGCCACGCCACCGGCGGGGCGCACCCGC 177
DNA region responsible for "activation" of melC1-p
GCCAAAGACCACCGGAAGGGACGTCCGCTCGGAAAGGAATTGCCCCTTCCGCCCGTCGGCGAGGACCGCCGCGAGCAAGATCATCTTTGTTCAACATTGCACGACAGATCATTAATTGTC 297
melC1-p
rbs
SphI
rbs
CGGATCGCGGCCAACCGGTCCGGGCCGATTTCTCCCCTTCTCCTCCGGTCGATAGGTATGCGGGGTCGTCAACCCAACGCACCCCAGGAGGTCCCGCATGCGGAGGGGTGCCTCATGAGC 417
(doxA) fM S
(oligopeptide) (fm r r g a s *)
SalI
GGCGAGGCGCCGCGGGTGGCCGTCGACCCGTTCTCGTGTCCCATGATGACCATGCAGCGCAAACCCGAGGTGCACGACGCATTCCGAGAGGGGGGCCCCGTCGTCGAGGTGAACGCCCCC 537
G E A P R V A V D P F S C P M M T M Q R K P E V H D A F R E A G P V V E V N A P
GCGGGCGGACCCGCCTGGGTCATCACCGATGACGCCCTCGCCCGCGAGGTGCTGGCCGATCCCCGGTTCGTGAAGGACCCCGATCTCGCGCCCACCGCCTGGCGGGGGGTGGACGACGGT 657
A G G P A W V I T D D A L A R E V L A D P R F V K D P D L A P T A W R G V D D G
BspEI
CTCGACATCCCCGTTCCGGAGCTGCGTCCGTTCACGCTCATCGCCGTGGACGGTGAGGACCACCGGCGTCTGCGCCGCATCCACGCACCGGCGTTCAACCCGCGCCGGCTGGCCGAGCGG 777
L D I P V P E L R P F T L I A V D G E D H R R L R R I H A P A F N P R R L A E R
ACGGATCGCATCGCCGCCATCGCCGACCGGCTGCTCACCGAACTCGCCGACTCCTCCGACCGGTCGGGCGAACCGGCCGAGCTGATCGGCGGCTTCGCGTACCACTTCCCGCTGTTGGTC 897
T D R I A A I A D R L L T E L A D S S D R S G E P A E L I G G F A Y H F P L L V
ATCTGCGAACTGCTCGGCGTGCCGGTCACCGATCCGGCAATGGCCCGCGAGGCCGTCGGCGTGCTCAAGGCACTCGGCCTCGGCGGCCCGCAGAGCGCCGGCGGTGACGGCACGGACCCT 1017
I C E L L G V P V T D P A M A R E A V G V L K A L G L G G P Q S A G G D G T D P
GCCGGGGACGTGCCGGACACGTCGGCGCTGGAGAGCCTTCTCCTCGAAGCCGTGCACGCGGCCCGGCGGAAAGACACCCGGACCATGACCCGCGTGCTCTATGAACGCGCACAGGCAGAG 1137
A G D V P D T S A L E S L L L E A V H A A R R K D T R T M T R V L Y E R A Q A E
TTCGGCTCGGTCTCCGACGACCAGCTCGTCTACATGATCACCGGACTCATCTTCGCCGGCCACGACACCACCGGCTCGTTCCTGGGCTTCCTGCTTGCGGAGGTCCTGGCGGGCCGTCTC 1257
F G S V S D D Q L V Y M I T G L I F A G H D T T G S F L G F L L A E V L A G R L
GCGGCGGACGCCGACGGGGACGCCATCTCCCGGTTCGTGGAGGAGGCGCTGCGCCACCACCCGCCGGTGCCCTACACGTTGTGGAGGTTCGCTGCCACGGAGGTGGTCATCCGCGGTGTC 1377
A A D A D G D A I S R F V E E A L R H H P P V P Y T L W R F A A T E V V I R G V
CGGCTGCCCCGCGGAGCGCCGGTACTGGTGGACATCGAGGGCACCAACACCGACGGCCGCCATCACGACGCCCCGCACGCTTTCCACCCGGACCGCCCTTCGAGGCGGCGGCTCACCTTC 1497
R L P R G A P V L V D I E G T N T D G R H H D A P H A F H P D R P S R R R L T F
GGCGACGGGCCGCACTACTGCATCGGGGAGCAGCTCGCCCAGCTGGAATCGCGCACGATGATCGGCGTACTGCGCAGCAGGTTCCCCCAAGCCCGACTGGCCGTGCCGTACGAGGAGTTG 1617
G D G P H Y C I G E Q L A Q L E S R T M I G V L R S R F P Q A R L A V P Y E E L
CGGTGGTGCAGGAAGGGGGCCCAGACAGCGCGGCTCACTGACCTGCCCGTCTGGCTGCGTTGATGGGCCGACCGCGACCCGGCACGGGACCGCCCACCGCCCATCGCGCGGTGGGCGGTC 1737
R W C R K G A Q T A R L T D L P V W L R *
CCGTGCCGGTCGCCCGGTGCGGTCCTCTCCCGACGCTCGCTCCCCCTGTGACTTTCTCACATCGAGACGTGACGAAATAATCCCAGCAAGTGCCATGCACACTTTCATGGCGGACATTCA 1857
rbs
CTTGCGAGGATGGAGTGAGCACACGGGGCCGCCCGAGACACCCTACGGCCGCCGGAAGTATGCCACCTGTTGACGCGAATGGAACGCCACAGAGGGAGCACCGGCAATGCAGATCAATAT 1977
(dauI) fM Q I N M
CTTGGGCCCGCTCGTTGCACATCACAATGGCACGTCGGTGACCCCGATAGCCAGAAAACCCCGGCAGGTATTCTCACTGCTCGCTCTTCAGGCAGGAACCGTCGTTCCGGTCCCCGCGCT 2097
L G P L V A H H N G T S V T P I A R K P R Q V F S L L A L Q A G T V V P V P A L
SstI
CATGGAGGAGCTC 2110
M E E L...

FIGURE 11

pANT144 - Used for bioconversion of
e-Rhodomycin D to 13-Deoxycarminomycin
and 13-Deoxydaunomycin



* Wild-type promoter

FIGURE 12